Epigenetic Deregulation in Rheumatoid Arthritis Synovial Fibroblasts

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Universität Zürich

von

Emmanuel Karouzakis

aus

Griechenland

Promotionskomitee

Prof. Dr. Max Gassmann (Vorsitz)
Prof. Dr. Steffen Gay (Leitung der Dissertation)
Prof. Dr. Adriano Fontana
Prof. Dr. Josef Jiricny

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Zusammenfassung

Die Rheumatoid Arthritis (RA) ist eine chronische Autoimmunerkrankung mit fortschreitender Zerstörung der Gelenke. In der Pathogenese der Erkrankung spielen zwei zelluläre Kompartimente eine wesentliche Rolle. Das eine beinhaltet aktivierte Immunzellen wie T-Zellen, B-Zellen und Makrophagen. Kennzeichnend für diese Gruppe ist das Freisetzen von entzündlichen Botenstoffen (Zytokine) wie Interleukin (IL)- 1 beta, IL-6 und „tumor-necrosis-factor“ (TNF) alpha sowie Autoantikörpern von Plasmazellen. Das andere umfasst die aktivierten synovialen Fibroblasten (RASF), Osteoklasten und Chondrozyten, alles Effektorzellen, die durch Aktivierung zur Zerstörung der Gelenke beitragen.

Bis heute konnte kein genetischer Polymorphismus aufgezeigt werden, der den aktivierten Phänotyp dieser synovialer Fibroblasten ausreichend erklärt. Unsere Hypothese ist, dass epigenetische Modifikationen, insbesondere veränderte DNA Methylierung zur Aktivierung dieser Zellen beitragen und so die Zerstörung von Knorpel- und Knochensubstanz herbeiführen.

Wir zeigen hier erstmalig eine Untersuchung des Methylierungsmusters synovialer Fibroblasten von Patienten mit RA. In in vivo Untersuchungen wiesen synoviale Gewebe von Patienten mit RA eine signifikante Hypomethylierung in den untersuchten Zellkernen auf im Vergleich zu Geweben von Patienten mit Osteoarthritis (OA). Und das betrifft insbesondere die synovialen Fibroblasten wie bei in vitro Untersuchungen isolierter Zellkerne gezeigt werden konnte (Chapter 2, Figure 1).

Inflammatorische Zytokine wie TNF alpha und IL-1 beta hatten einen positiven Einfluss auf den Zellzyklus und gleichzeitig führten sie zu einer reduzierter Menge an 5-Methylcytosin in den Zellkernen der untersuchten Zellen. Wir zeigen hier eine reduzierte Expression von DNA-Methyltransferase 1 (DNMT1) in RASF, die möglicherweise zur progressiven Hypomethylierung dieser Zellen beiträgt. In diesem Zusammenhang unterstützen proinflammatorische Zytokine den Prozess der DNA Hypomethylierung durch Aktivierung der Zellproliferation, sie sind aber nicht die Ursache des niedrigen basalen Levels an DNMT1 in RASF. Darüber hinaus konnten wir aufzeigen, dass repetitive Sequenzen wie LINE-1 in

RASF demethyliert sind, was die Hypothese einer globalen Hypomethylierung in RASF unterstützt.

Um die für den aktivierten Phänotyp synovialer RA Fibroblasten verantwortlichen Gene zu identifizieren, wurden synoviele Fibroblasten von gesunden Probanden über einen Zeitraum von 2 Monaten mit nicht toxischen Mengen des DNMT1-Inhibitors 5-azacytidine (5-azaC) behandelt, und anschießend eine Genexpressionsanalyse durchgeführt. Wir konnten zeigen, dass mehr als die Hälfte der so überexprimierten Gene mit denen übereinstimmen, die mit der Pathogenese der RA assoziiert wurden. Dies sind Gene, die zur Gelenkzerstörung beitragen wie Matrix-Metalloproteinasen (MMPs), Integrine und viele weitere, die im Kapitel 2 aufgeführt sind (Suppl. Tables 1,2).

In Kapitel 3 zeigen wir die Untersuchung eines spezifischen Gens mit verändertem Methylierungsmuster in RASF. Aktivierte RASF exprimieren unterschiedliche Chemokine, die so weitere Immunzellen in die betroffenen Gelenke locken. CXCL12 (SDF-1α) ist eines dieser Chemokine, welches in RASF überexprimiert wird. Wir konnten nachweisen, dass im Vergleich zu OASF, in RASF der Promotor von CXCL12 weniger methyliert ist. Weiterhin korrelierte die Expression von CXCL12 auf mRNA Level in diesen Zellen signifikant mit der Hypomethylierung im CXCL12 Promotor. Die durch Hypomethylierung verstärkte Expression von CXCL12 in RASF induzierte dann in den Zellen eine verstärkte Expression von MMPs durch die Bindung an den dazugehörigen Rezeptor CXCR7.

Wir beschreiben hier einen endogenen Aktivierungsmechanismus in RASF, der zur progressiven Zerstörung der Gelenke beiträgt. Basierend auf unserer Daten verursacht eine veränderte globale und genspezifische DNA Methylierung den aktivierten invasiven Phänotyp der RASF.

Summary

Rheumatoid arthritis is a chronic autoimmune disease involving destruction of affected joints. Two cellular compartments are involved in the pathogenesis of RA. The first one involves activated T cells, B cells and macrophages. They secrete a variety of pro-inflammatory cytokines such as interleukin-1beta, IL-6 and tumor necrosis factor alpha (TNFalpha) as well as a variety of autoantibodies. The second compartment involves activated rheumatoid arthritis synovial fibroblasts (RASF), osteoclasts and chondrocytes that are the effector cells of joint destruction. The thesis focuses on the epigenetic mechanisms leading to the activated phenotype of RASF.

Since to date no genetic polymorphism can explain the hyperactive phenotype of synovial fibroblasts, we hypothesised that epigenetic modifications, particularly impaired DNA methylation, can cause the activation of synovial fibroblasts and lead to joint destruction. For the first time, the methylation status of cells was analysed in the synovium of patients with rheumatoid arthritis (RA). RA synovial tissues were found to have hypomethylated nuclei (Chapter 2, Figure 1). Especially RASF had low amounts of 5-methylcytosine. Pro-inflammatory cytokines such as TNF alpha and IL-1 beta induced cell cycle progression and reduced further the amount of 5-methylcytosine in the nuclei. We reported a deficiency of DNA methyltransferase 1 (DNMT1) in RASF that can be an important factor involved in the progressive demethylation. In this context, pro-inflammatory cytokines favor DNA hypomethylation by increasing the rate of cell proliferation; however, they were not responsible for low basal levels of DNMT1. Furthermore, repetitive sequences such as LINE-1 were demethylated in RASF, supporting the hypothesis of an active global hypomethylation in these cells. To mimic the chronic hypomethylation state of synovial fibroblasts and identify gene targets, normal synovial fibroblasts were treated over a long period of time (2 months) with a non-toxic dose of the DNMT1 inhibitor 5-azacytidine (5-azaC) and a gene expression analysis was performed. More than half of the genes that were found to be overexpressed by this treatment were previously associated with the pathogenesis of RA. These included genes associated with joint destruction such as matrix metalloproteinases (MMPs), integrins and others summarized in Chapter 2 (Suppl. Tables 1,2).

Finally, a specific gene target that may have an impaired DNA methylation in RASF was analysed in Chapter 3. Activated RASF secrete chemokines that attract a variety of inflammatory cells into the joint. CXCL12 (SDF-1α) is a chemokine overexpressed and secreted by RASF. We reported that the promoter of CXCL12 is less methylated in RASF than in osteoarthritis synovial fibroblasts (OASF). The mRNA expression of CXCL12 significantly correlated with the CXCL12 promoter methylation. The upregulation of CXCL12 could stimulate RASF to produce more MMPs via the receptor CXCR7.

Thereby, we describe an endogenously activated pathway in RASF which promotes joint destruction. In conclusion, this study confirms the hypothesis that global and gene specific DNA methylation alterations are responsible for the activated phenotype of RASF.

Abbreviations

5-azaC	5- azacytidine
5-MeC	5-methylcytosine
Anti-CCP	anti-cyclic citrullinated peptide
AP-1	activator protein 1
cDNA	complementary DNA
CK	cathepsin K
CRP	C-reactive protein
CXCL12	chemokine (C-X-C motif) ligand 12 (SDF-1)
dCt	delta Ct
DMARDs	disease modifying antirheumatic drugs
DNMT-1	DNA methyltransferase 1
FACS	fluorescence activated cell sorting
HDAC	histone deacetylases
HPLC	high Performance Liquid Chromatography
ICF	immunodeficiency, centromere instability and facial anomalities syndrome.
ICR	imprinting control region
IL-1β	interleukin 1 beta
MeCP2	methylated CpG binding protein 2
MMP	matrix metalloproteinases
MTX	methotrexate
NSAIDs	nonsteroidal anti-inflammatory drugs
NSF	normal synovial fibroblasts
OA	osteoarthritis
ORF1	open reading frame 1
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RA	rheumatoid arthritis
RASF	rheumatoid arthritis synovial fibroblasts
RFs	rheumatoid factors
SAM	S-adenosylmethionine

Abbreviations

SCID mice	severe combined immunodeficiency mice
SDF-1	stromal derived factor 1 (CXCL12)
SF	synovial fibroblasts
siRNA	small interfering RNA
TNFα	tumor necrosis factor alpha
TSA	trichostatin A

Chapter 1: Introduction

The introduction is adapted from the following publications and books:

Karouzakis E., Gay R.E., Gay S., Neidhart M. Epigenetic deregulation in rheumatoid arthritis. Advances in Experimental Medicine and Biology 2011; 711:137-149.

Karouzakis E., Gay R.E., Gay S., Neidhart M. Epigenetic control in rheumatoid arthritis synovial fibroblasts. Nature Reviews Rheumatology 2009; 5(5):266-72.

Karouzakis E., Jüngel A., Gay R.E., Kolling C., Gay S., Neidhart M. Epigenetic approaches to the study of the pathogenesis of rheumatic diseases. European Musculoskeletal Review 2008; 3:41-43.

Karouzakis E., Neidhart M., Gay R.E., Gay S. Molecular and cellular basis of rheumatoid arthritis joint destruction. Immunology Letters 2006; 106(1):8-13.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder of unknown etiology that is associated with progressive joint destruction. RA is described as an autoimmune disease and is characterised by polyarticular pain, swelling, morning stiffness, malaise and fatigue. The disease does not have specific features and develops over a long period of time. Arthritis affects the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of both hands. This symmetry is the most characteristic early clinical feature. Classification criteria for RA were drafted in 1956 by the American College of Rheumatology (ACR) and revised in 1987 in order to provide guidelines for clinical trials [1]. Also, rheumatologists, in order to assess the effect of different medications in the treatment of RA, developed the ACR scoring system. ACR 20 means 20 % improvement based on a scale that includes number of affected joints, inflammatory markers, pain and disability scoring. Terms like "ACR 20", "50" or "70" which mean 20% to 70 % significant improvement obtained with a given drug are usually found in the literature.

1. Etiology [adapted from Karouzakis E. et al. Immunology Letters 2006; 106(1):8-13, and Kelley's Textbook of Rheumatology; 2009]

RA affects 0.8-1.0 % of the population [2]. RA appears less common in Asia and Africa than in United States and Europe. In young adults, the disease is predominantly found in women. Men are less affected than women, but the occurrence of the disease increases with age and reaches the same proportion between genders in the elderly. The etiology of RA remains still unknown. It has been hypothesised that infectious agents such as viruses and bacteria cause a chronic inflammatory response that targets components of the joint [3].

Different research data showed evidence of autoimmunity, the most obvious being the production of autoantibodies and the association with specific HLA subtypes. Thus, the genetic predisposition to RA of individuals with HLA-DRB1 "shared epitope" has raised the possibility of a specific autoantigen that triggers a T cell clonal response [4]. The susceptibility to RA is associated with the epitope glutamine-leucine-arginine-alanine-alanine (QKRAA) that is found in HLA-DR4, DR14 and some DR1beta chains. However, experiments that tried to elute peptides from the pocket of this RA susceptibility allele did not reveal a specific antigen for RA. The shared epitope might be not the most important and is certainly not a unique risk factor for RA. Thus, in large studies of patients with undifferentiated inflammatory arthritis, the progression to RA occurred regardless of the

HLA-DR genotype. On the other hand, about 50% of RA patients are positive for anti-cyclic citrullinated peptide (anti-CCP) autoantibodies. Patients with the susceptibly HLA-DR phenotype and with anti-CCP antibodies develop a disease with greater severity. Further research regarding the genetic influence on the disease led to the investigation of single nucleotide polymorphisms in the promoter regions of various genes. Several polymorphisms have been described for inflammatory cytokines and chemokines including tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β) and RANTES. Gene polymorphisms that are independent from HLA genotype and pro-inflammatory cytokine levels are peptidyl arginase deiminase (PADI) and PTPN22 [5, 6]. PADI genes include four alleles (PADI1 to PADI4) and PADIs are responsible for the post-translation modification of arginine to citrulline. PTPN22 is responsible for the phosphorylation status of several kinases that have important roles in T cell activation.

Environmental factors, such as smoking, have also been implicated in the development of RA. The influence of smoking is not clearly known [7]. Smoking increases the risk of developing anti-CCP antibodies in carriers of the HLA-DR shared epitope [8].

Examination of ancient skeletons in Europe failed to detect the onset of RA until end of the 18^{th} century with the beginning of industrialisation. In contrast, RA was evident in Native Americans thousands of years ago. A possible hypothesis is that the disease migrated from the New World to the Old World with trading, exploration trips and wars. Perhaps an infectious agent was responsible for the propagation. Indeed, different infectious agents have been associated to the pathogenesis of RA, including mycobacteria, E. coli, Epstein Barr virus, parvovirus and retroviruses [9]. These infectious agents could be involved in the phenomenon of molecular mimicry or in the alteration of the activity of cells involved in innate and adaptive immunity.

2. Pathogenesis [adapted from Karouzakis E. et al. Immunology Letters 2006; 106(1):8-13, Kelley's Textbook of Rheumatology; 2009]

RA is a disease of the musculoskeletal system. Synovial joints are mainly affected by the disease. A typical example of a normal synovial joint is shown in Figure 1. Characteristic structures include two bones joined by a fibrous capsule and ligaments to support the joint. The synovial membrane or synovium is located in the inner part of the fibrous capsule and surrounds the joint everywhere, except the articular cartilage. The inner surface of the membrane is usually smooth and folded into numerous processes called villi. The synovium is

abundantly supplied with blood vessels, nerves, and lymphatics; it provides immunological protection and produces synovial fluid. The synovial membrane consists of two cell types: synovial fibroblasts (SF) and macrophages. The SF produce a long chain sugar polymer called hyaluronan that is a component of the synovial fluid together with a molecule called lubricin, which lubricates the joint surfaces. The synovial fluid provides a liquid environment for the joint surfaces and nutrients for chondrocytes enclosed in the cartilage. The healthy hyaline cartilage is a connective tissue consisting of proteoglycans, collagen, glycoproteins and water. Chondrocytes are the specialised cells for the homeostasis of cartilage. The macrophages guard the synovial fluid from pathogens.

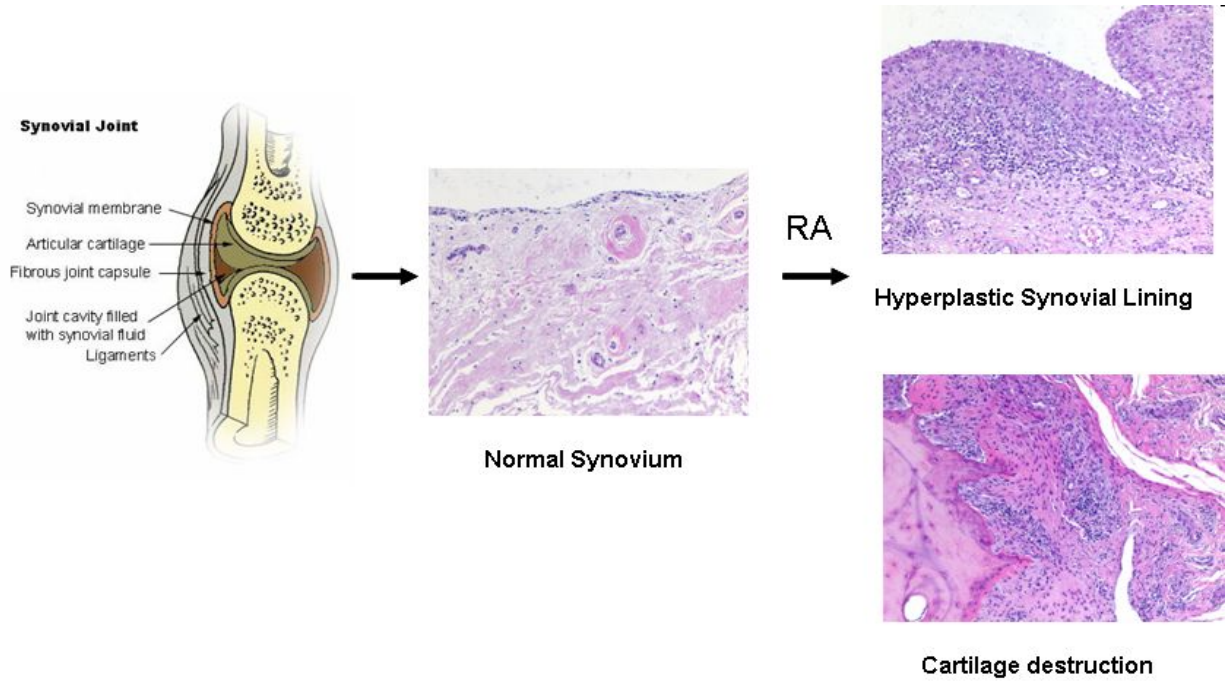

Figure 1: Characteristics of a knee synovial joint in normal conditions and rheumatoid arthritis (RA) patients [retrieved from the web site of Prof. Michel Neidhart- http://epigenetics.ch].

In RA, the synovial tissue is hypertrophic and inflamed. It contains diverse cell populations, including T cells, B cells, macrophages, dendritic cells, neutrophils, mast cells and SF. Microscopic examination of synovial tissues from patients with RA showed that there is an increased thickness of the synovial lining and an infiltration of cells around small blood vessels, leading to the characteristic sign of synovial hyperplasia. The rheumatoid endothelial cells have an altered function and express various adhesion molecules that facilitate the entry

of cells into the tissue. Memory T lymphocytes (CD4+, CD45RO+) are the major infiltrating cells in the rheumatoid synovium [10]. They are found in close proximity with dendritic cells, SF and macrophages and secrete Th1-like pro-inflammatory cytokines, such as interferon-gamma (INFγ).

As a result of continuous T cell activation and cytokine secretion, macrophages are in turn activated and release TNFα and IL-1β [11]. These pro-inflammatory cytokines activate SF to secrete cartilage degrading enzymes, such as matrix metalloproteinases (MMPs).

Activated B cells produce a number of autoantibodies, such as rheumatoid factors (RFs) and anti-CCP autoantibodies in the synovium. RFs are typically immunoglobulins (Ig)M antibodies that bind the Fc fragment of IgGs. They are considered a late marker of the disease and appear in the sera of 70-80% of patients with RA. Autoantibodies to CCP have been identified as markers for RA. Interestingly, these autoantibodies can be detected several years before the onset of the disease [12]. Autoantibodies, in particular RFs, form immune complexes that can activate, complement and increase inflammation. Patients with RA develop germinal centre structures in the synovial tissue [13].

In addition, an increased number of mast cells have also been found in the rheumatoid synovium and shown to release granules that contribute to ongoing inflammation. Osteoclasts are also found at sites of bone destruction. They are derived from myelomonocytic precursor cells with the help of T cells and SF that express the receptor activator of NFkB ligand (RANKL) [14]. Lymphocytes, macrophages, SF and osteoclasts secrete a large variety of products that cause the pathological features of rheumatoid synovium including inflammation, hyperplasia, infiltration, activation, proliferation, as well as cartilage and bone destruction [15].

3. Treatment [adapted from Kelley's Textbook of Rheumatology; 2009]

Most therapies today are based on the use of Nonsteroidal Anti-inflammatory Drugs (NSAIDs), Disease Modifying Antirheumatic Drugs (DMARDs) and biologic agents which mainly target the chronic inflammation [16].

NSAIDs have the ability to suppress the synthesis of prostaglandins by inhibiting the cyclooxygenase enzymes (COX-1 and COX-2). The expression of these enzymes is induced during inflammation. The most popular DMARD drug for RA treatment is methotrexate. At low doses, it has immunomodulatory and anti-inflammatory effects. Also, another successful DMARD drug is leflunomide that inhibits the synthesis of pyrimidines and effects T cell proliferation.

Monoclonal antibodies such as Infliximab and Adalimumab as well as soluble receptors like Etanercept that inhibit TNFα, have been used successfully in the treatment of RA [17]. In addition, Abatacept is a recombinant CTLA4Ig that downregulates T cell activation by interfering with T cell signalling [18]. Also, therapies targeting the B cells have been exploited. Clinical trials with Rituximab (anti-CD20) have shown some impressive results in the treatment of RA [19].

However, the biological therapies mentioned above which target only the immune system cells do not have an ACR 70 response over 60%. All current therapies ignore the role of synovial fibroblasts in the pathogenesis of RA.

4. Activated rheumatoid arthritis synovial fibroblasts [adapted from Karouzakis E. et al. Immunology Letters 2006; 106(1):8-13, and Karouzakis E. et al. Nature Reviews Rheumatology 2009; 5(5): 266-72]

Rheumatoid arthritis synovial fibroblasts (RASF) are mainly found in the synovial sublining of joints which is multilayered and hyperplastic. Activated RASF display an aggressive and invasive behaviour that resembles those of metastatic cancer cells (Figure 3). They have characteristic morphological features such as a round shape and a large pale nuclei with prominent nucleoli [20]. The most characteristic functional feature is the ability of RASF to adhere to cartilage and to initiate the degradation of extracellular matrix (ECM) components. Many observations support the notion that the activated phenotype of RASF is an intrinsic property of these cells. The clearest evidence came from an animal model using severe combined immunodeficiency mice (SCID) in our laboratory [21]. In this model, SFs were implanted together with human cartilage under the renal capsule of SCID mice for 60 days. During this period, RASF attached to and invaded the cartilage in a similar way as observed in the human joints. Most important is the fact that the invasion of RASF occured without any support from the immune system. A recent study using a similar model has further proposed

that RASF are able to migrate from an affected joint to a distant healthy joint and are responsible for the symmetrical distribution of the disease [22, 23]. Indeed, the RA synovial fluid contains "floating" fibroblast-like cells that are able to destroy cartilage. This is not found in other joint diseases, such as osteoarthritis (OA) [24].

Synovial hyperplasia could be due to an increased rate of proliferation of the RASF. This concept is supported by the increased expression of transcription factors, markers of proliferation and growth factors, including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGFbeta) [25-28]. Proto-oncogenes including ras, myc, myb involved in the regulation of the cell cycle, transcriptional regulation, or acting as growth factors in a variety of cells are expressed abundantly in RASF, especially at sites of invasion [29]. Macrophage inhibitory factor (MIF) has been shown to cause the proliferation of RASF through the ERK MAP kinase pathway [30]. MIF knock out mice revealed a reduced rate of proliferation and high p53 expression [31]. However, RASF did not proliferate more than other SF *in vitro*. In addition, Seemayer et al. and Kunzler et al. provide evidence that the invasive behaviour of RASF cell is independent from their proliferation [32, 33].

Another property of the RASF that might contribute to synovial hyperplasia is the decreased rate of apoptosis [34], especially at sites of synovial invasion into cartilage and bone. Although Fas ligands are present in synovial fluid, the cells appear resistant to apoptosis [35]. Several studies have examined the expression of anti-apoptotic molecules in RASF. FLICE like inhibitory protein (FLIP) is expressed by RASF at sites of invasion into cartilage and bone [36]. It can inhibit the pro-caspase 8 and interfere with the apoptosis signalling pathway. FLIP expression is induced by TNFα and siRNA silencing of FLIP sensitises the cells to apoptosis [37].RASF express high levels of SUMO-1 protein that protects the cells from apoptosis [38]. A recent study suggested that SUMO-1 inhibits apoptosis in RASF indirectly by modifying the nuclear promyelocytic leukemia (PML) that traps the pro-apoptotic molecule DAXX in the PML nuclear bodies [39]. Moreover, the stimulation of RASF with MIF reduces the rate of apoptosis in RASF. MIF knock out mice have reduced severity of antigen induced arthritis due to high expression of p53 and apoptosis in the synovium [31]. Furthermore, the novel molecule synoviolin, an E3 ubiquitin ligase, has been described as another important regulator of proliferation and apoptosis in RA [40].

Thus, both proliferation and resistance to apoptosis appear to contribute to the synovial hyperplasia in RA. It is important to note, however, that it is currently thought that the migration of inflammatory cells into the synovial tissue predominantly contributes to this hyperplasia.

Activated RASF maintain the ongoing local inflammatory immune response in the joint (Figure 2). The production and secretion of interleukin-15, -16, -17 and CXCL12 /SDF-1α (stromal cell derived factor 1 α) from RASF appear in great part responsible for T cell activation [41, 42]. In addition, CXCL12 may promote neoangiogenesis. CXCL13 has been shown to promote the migration of B cells into the synovium [43, 44]. In turn, B cells express cytokines such as lymphotoxins α and β, which activate follicular dendritic cells, attract T cells and further organise the germinal centers. In addition, RASF show a hyperreactive response to the proinflamamtory cytokines TNFα and IL-1β. The activation characteristics of RASF are dependent on multiple transcription factors such as Nuclear Factor-kβ (NF-kβ) and Activator protein-1 (AP1), the p38 stress activated MAP kinases (MAPK) and PI-3 kinase. These signalling pathways in RASF reflect the maintenance of a chronic pro-inflammatory cytokine milieu in the synovium that in turn triggers the production of catabolic enzymes.

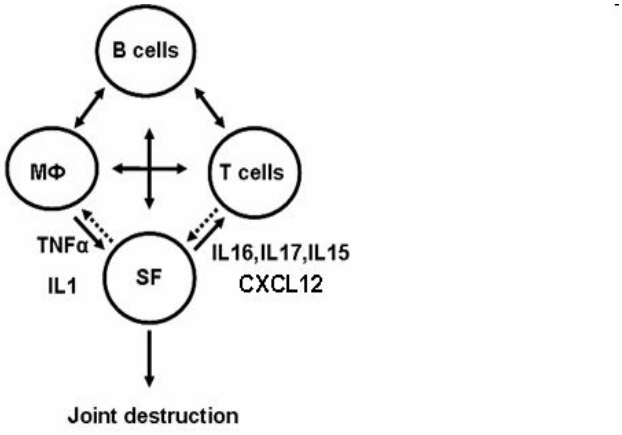

Figure 2: Interaction between cells of the immune system and synovial fibroblasts mediating joint destruction [adapted from Karouzakis E. et al. Immunology Letters 2006; 106(1):8-13]

The expression of members of the interleukin-6 family such as IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OSM) are expressed by RASF at the site of invasion [45]. Another critical modulator of inflammation is prostaglandin E2 (PGE2). RASF secrete high amounts of PGE2 in the inflammatory synovium [46].

The destructive potential of RASF is reflected by their ability to attach to certain cartilage components. Galectin-3 is triggered in RASF upon attachment to type VI collagen or cartilage oligomeric matrix protein (COMP) [47]. The adhesion to COMP occurs via the αVβ3 integrin receptor. The adhesion to fibronectin rich cartilage areas is maintained by overexpression of beta integrin receptors (VLA-3, VLA-4, VLA-5) [48]. Furthermore, RASF express other adhesion molecules such as VCAM-1 and CS1 fibronectin isoform, which bind to lymphocyte integrin receptors and contribute to the maintenance of the inflammatory response [49]. Cadherin 11 is also expressed by RASF and in vitro facilitates the invasion of RASF into the cartilage [50].

CD44, the hyaluronic acid receptor, and its isoforms are another group of molecules associated with the destructive potential of RASF. In this regard, it is interesting to note that anti-CD44 antibodies inhibit the RASF-mediated destruction of cartilage [51]. Possibly, certain CD44 isoforms (such as CD44v8-v9) are specific for the invasive behaviour of RASF [52].

The degradation of the extracellular matrix is caused by the action of matrix metalloproteinases (MMPs) [53]. It is known that RASFs secrete MMP-1, MMP-3 and MMP-13 [54]. In addition, RASF produce MMP-14 and MMP-15 which belong to a family of membrane specific MMPs, called MT-MMPs, which activate MMP-2 and MMP-13 [55]. Another important group of extracellular matrix (ECM) degrading enzymes are cathepsins B, K and L [56]. These enzymes have been shown to be produced by RASF at sites of invasion and thought to contribute significantly to the joint damage.

Bone destruction is mainly caused by osteoclasts. In the synovial fluid of RA patients, large amounts of RANKL have been detected. RANKL, together with GM-CSF, promotes the differentiation of osteoblasts and monocyte progenitor cells into osteoclasts [14]. Most

interesting, RASFs secrete RANKL at sites of invasion [57], suggesting a link between activation of SF and osteoclastogenesis.

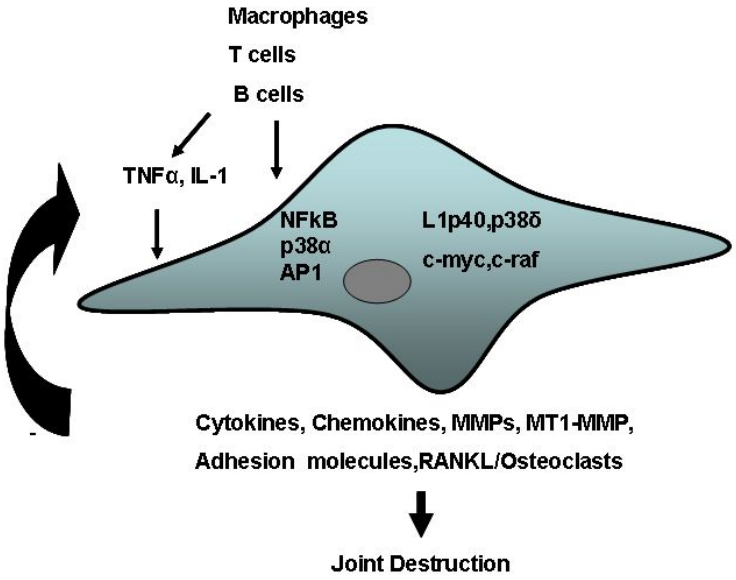

Figure 3: Characteristics of activated rheumatoid arthritis synovial fibroblast (RASF) [adapted from Karouzakis E. et al. Immunology Letters 2006; 106(1):8-13]

Retroviral-related sequences have been speculated as the etiologic agents for RA [58]. LINE-1 (L1) retrotransposons replicate genome repetitive elements that are similar to retroviruses, but lack the env gene [59]. The sequence of L1 retrotransposons contains two open reading frames. Open reading frame 2 (ORF2) encodes for a 150kd reverse transcriptase with endonuclease activity that is essential for retrotransposition. Open reading frame 1 (ORF1) transcribes for a 40 kd protein (p40). L1-p40 has been reported in the pathogenesis of cancer [60]. Neidhart *et al* described the expression of L1-p40 in synovial tissue, particularly at sites of joint destruction [61]. The L1-p40 protein normally acts as a cis-activator of the L1-p150 protein, and in addition it has been hypothesized that it might act as a trans-activator, triggering the expression of genes involved in the activation of RASF. Thus, in L1-p40-transfected RASF, p38 delta MAPK, c-met protooncogenes (the receptor for hepatocyte growth factor) and galectin-3 binding protein were upregulated [61, 62]. Interestingly, the expression of L1-p40 was localised at sites of invasion and is thought to play a major role in the upregulation of p38δ MAPK. The LINE-1 family of retrotransposons have a short internal promoter, the transcriptional activityof which is regulated by DNA methylation [62]. The increased activity of L1-p40 in RASF was associated with genomic hypomethylation [61, 62]. This was the first evidence of an epigenetic abnormality in the genome of RASF.

5. Epigenetics [adapted from Karouzakis E. et al. European Musculoskeletal Review 2008, 3: 41-43, Karouzakis E. et al. Nature Reviews Rheumatology 2009; 5(5): 266-72, and Karouzakis E. et al. Advances in Experimental Medicine and Biology 2011, 711: 137-149]

Epigenetics is an area of research that, in rheumatology, has only very recently been explored. It regards changes in gene expression that occur without a change in the DNA sequence. Epigenetics include DNA methylation and histone modifications (Figure 4). The interplay between these two processes controls the accessibility of chromatin to transcription factors.

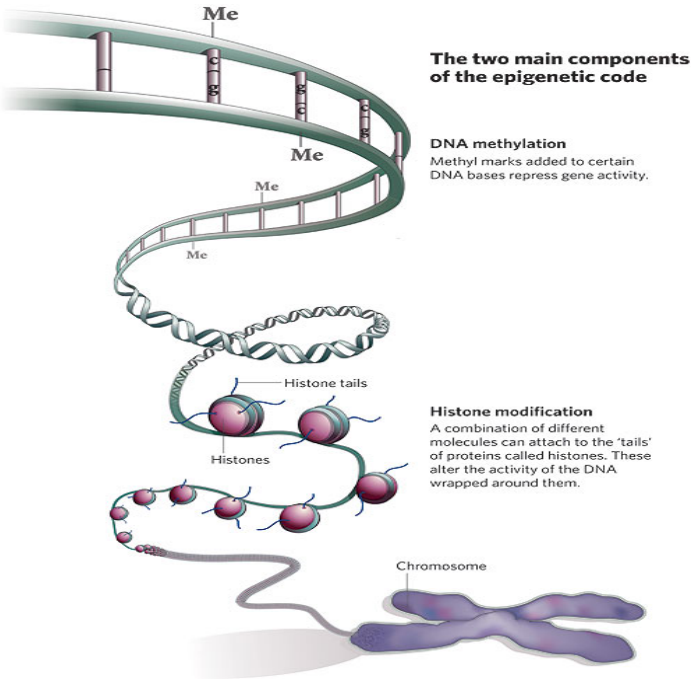

Figure 4: The two main components of the epigenetic code [63]: DNA methylation and histone modifications.

DNA methylation involves the addition of a methyl group in the 5' position of the cytosine base pair ring. It is the oldest epigenetic modification that is found in bacteria, fungi and plants. The organisms that do not methylate their own DNA are relatively small and include C.elegans and S.cerevisiae. DNA methylation plays important role in the developmental process. In embryonic development, DNA methylation during fertilisation of female and male genomes undergoes extensive demethylation [64]. New methylation marks are established by *de novo* methylation. Mice that have lost DNA methylation are embryonic lethal during organ development (Figure 5) [65]. Also, changes in DNA methylation are associated with

pathological conditions such as different types of cancer [66]. In humans, DNA methylation is found in the sequence context of 5`-CpG-3` [67]. Distinct patterns of DNA methylation are established during development. These patterns are stable and transmitted to next generations. Regulatory sequences such as promoters of housekeeping genes are kept methylation free. CpG islands are often associated with promoters of genes and have been thought as key factors in gene regulation. They are kept methylation free in somatic cells. However, different tumor suppressor genes have been shown to be silenced by promoter methylation of CpG islands [68]. The following criteria have been used to define a CpG island: G+C content of more than 55%, an observed vs. expected ratio of more than 0.65 and minimum size 500bp [69].

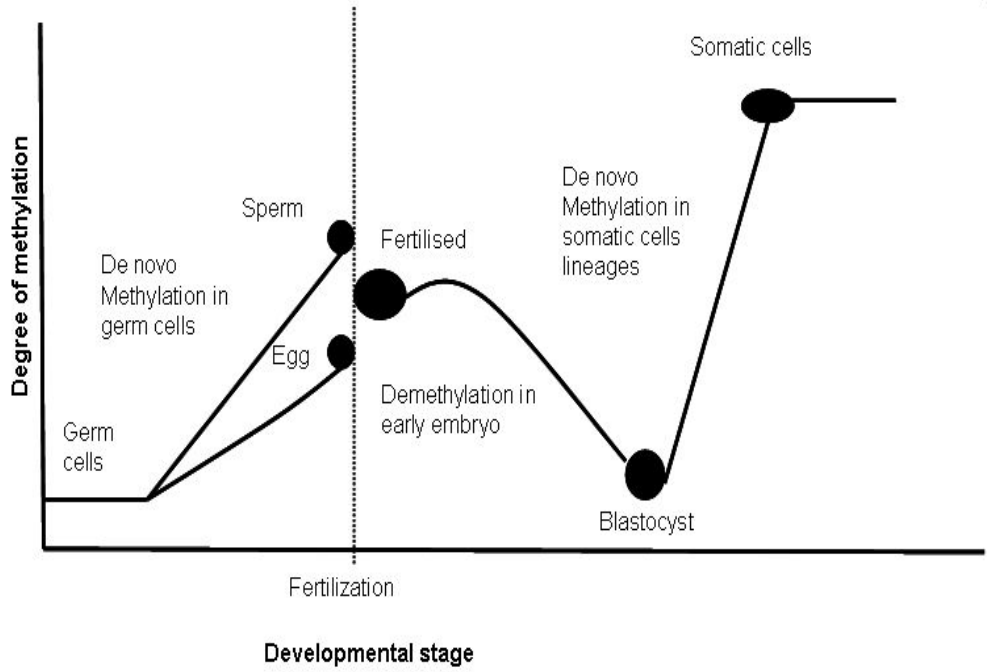

Figure 5: DNA methylation in development [70].

5.1 DNA methyltransferases [adapted from Jörg Tost Epigenetics book ref (84)]

DNA methylation is an enzymatic reaction catalyzed by DNA methyltransferase 1 (DNMT1) [71] (Figures 6 and 7). DNMT1 is responsible for copying marks of methylation in the context of CpG dinucleotides during somatic cell replication. It targets hemimethylated DNA and catalyses the transfer of a methyl group from S-adenosylmethionine (SAM) to the 5' prime end of the cytosine base pair [67]. The end product is 5-methylcytosine.

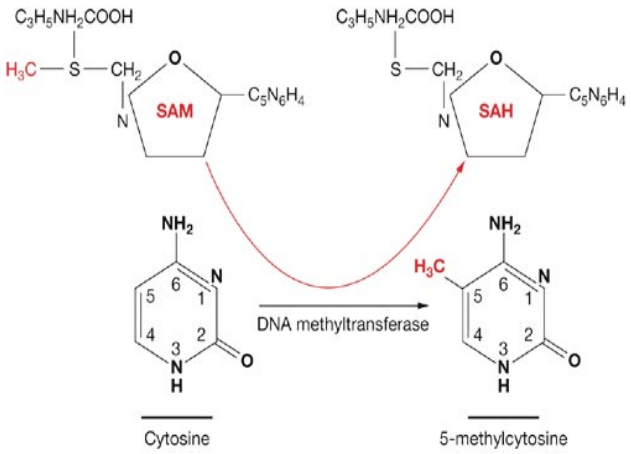

Figure 6: DNA methyltransferase catalyses the conversion of cytosine to 5-methylcytosine [72].

The full human DNMT1 cDNA was cloned and mapped in chromosome 19p13.2 [73]. It has 80% homology with the mouse at the mRNA level and 74% homology at the amino acid level. DNMT1 protein has two domains: regulatory and catalytic [73]. The regulatory domain is located at the N' terminal end of the protein and is responsible for all protein to protein interactions. It targets the protein in the replication fork through a proliferating cell nuclear antigen (PCNA) domain binding domain [74]. PCNA targets DNMT1 in the replication fork, but it does not affect its methylation activity. It also possesses a conserved cysteine rich CxxC region that interacts with histone deacetylases (HDAC1/2) and regulates transcriptional repression through chromatin modification [75]. DNMT1 is also associated with other proteins including retinoblastoma (Rb), p53, Sp1 and histone H3K9 methyltransferase SUV39H1 [76, 77]. The carboxylic terminal end of the protein contains the catalytic motifs IV and VI which are responsible for the enzymatic function of the protein. The motif IX for the ability of the DNMT1 to interact with DNA double helix and the I, X motif are associated with the binding of S-adenosylmethionine (SAM), the cell methyl's donor. DNMT1 is ubiquitously expressed with the highest expression levels in the brain, lung, heart and placenta. The expression of the protein increases during the S phase of the cell cycle. Proliferative cells express high levels of DNMT1 in order to maintain DNA methylation during replication [78]. The presence of DNMT1 is essential for normal embryonic development. DNMT1 knock out caused embryonic lethality in mouse embryos [79]. DNA demethylation in cell lines has been achieved *in vitro* by DNMT1 RNA interference and treatment with the chemotherapeutic agent 5-azacytidine which inhibits its enzymatic activity.

During embryonic development the DNA methyltransferases 3a and 3b (DNMT3A/B) are expressed and add the initially methylated CpG dinucleotides in the DNA. (Figure 7) The mouse and human sequences of DNMT3A/3B have 94%/98% homology [80]. They share a conserved catalytic domain with the other metyltransferases DNMT1 and DNMT2. In contrast to DNMT1 that requires the intramolecular interaction of N and C terminal domain, the DNMT3A/B requires only the catalytic subunit [81]. They are very important for the establishment of imprinted genes during embryonic development. Similar to DNMT1, these enzymes interact with different proteins that regulate DNA damage and histone modifications. In addition, the interaction of DNMT1 and another methyltransferase DNMT3L might enhance both of their activity [82].

DNMT2 is another member of the family that has a conserved catalytic methyltransferase motif but it does not methylate DNA *in vivo*[83]. This was shown in knock out embryonic stem cells that retain DNA methylation of their genome. In contrast to the nuclear localisation of the other methyltransferases, DNMT2A is located in the cytoplasm. It functions as a RNA methyltransferase that specifically methylates $tRNA^{asp}$ [83]. The function of this modification to date remains unknown.

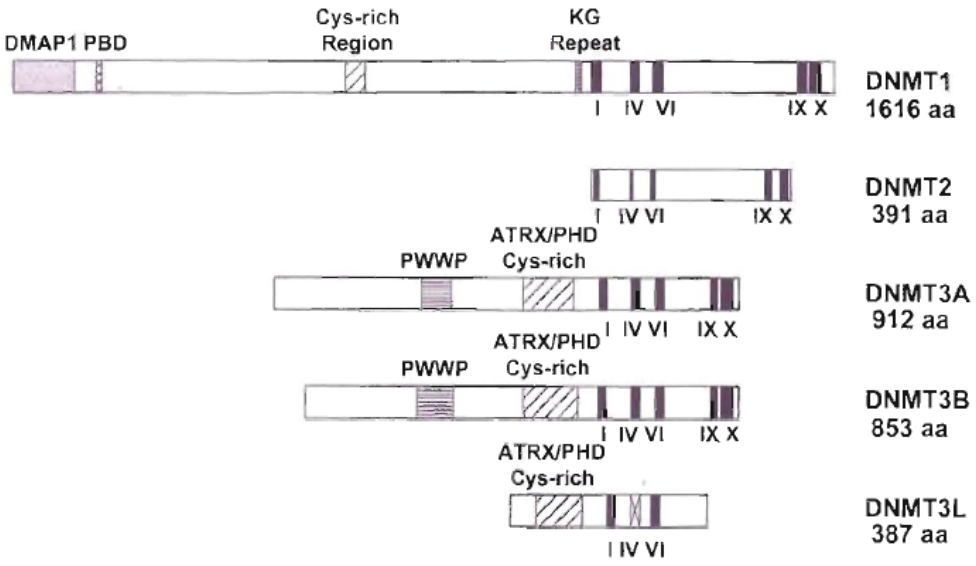

Figure 7: DNA methyltransferase family [84].

Defects in DNA methyltransferases have been implicated in tumorigenesis and developmental disorders. In cancer, epigenetic changes have been observed in a variety of tumors and include global hypomethylation and specific hypermethylation of tumor suppressor genes

promoters. Analysis of the expression of DNMT1 and DNMT3A showed an increased level in tumors compared to normal tissues [85]. Mutations of DNMT1 have been identified in colorectal cancer. Loss of p53 which regulates DNMT1 transcriptionally can cause abnormalities in DNA methylation [86]. Increase of DNMT1 does not always correlate with genomic hypermethylation. Progression of hepatocellular carcinoma (HCC) is followed by genomic hypomethylation and increased expression of DNMT1 [87]. However, in general, the increase of DNMT1 is associated with increased proliferation of tumor cells.

Immunodeficiency centromere instability and facial anomalies (ICF) syndrome is an autosomal recessive disease associated with abnormal DNA methylation and mutations in the catalytic domain of DNMT3B [88]. The disease causes immunodeficiency and involves hypomethylation of repetitive sequences and chromosomal instability due to hypomethylation of satellite repeats and the expression of genes on the X inactive chromosome. The immunodeficiency is caused by the reduction in immunoglobulin production.

5.2 Functional role of DNA methylation [adapted from Jörg Tost Epigenetics book ref (84)]

5.2.1 Transcriptional repression

DNA methylation represses the transcriptional regulation of genes. This mechanism is particularly important in embryogenesis and cell differentiation. Three mechanisms have been proposed (Figure 8).

A first mechanism is the inhibition of transcription factor binding to promoter sites with methylated CpGs. Several transcription factors such as AP1, NFkβ, cAMP response element binding (CREB), E2F recognise sequences that contain CpG islands or sequences with accumulated CGs and the methylation of such sites inhibits the binding of specific transcription factors [89].

A second mechanism is mediated by the direct binding of transcription repressors that recognise the methylated CpG motifs and inhibit binding of transcription factors and DNA polymerase II.

The third mechanism is associated with proteins that bind methylated DNA (such as MBD1, MeCP2, MBD3) blocking the interaction of promoters with transcription factors and DNA polymerase II. They also interact with histone deacetylases to change the chromatin structure [90].

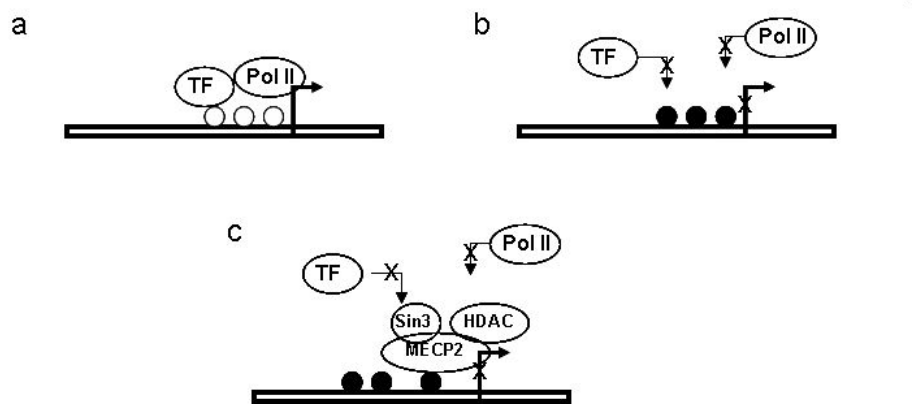

Figure 8: Mechanisms of transcriptional repression mediated by cytosine methylation: a) Active transcription. b) Repression by inhibiting transcription factors. c) Repression by MeCP2. ● 5-methylcytosine

MeCP2, as DNMTs, is important for embryonic development and binds to methylated CpGs with A/T-rich runs. It has two regulatory domains: a methyl CpG binding domain that targets the protein to methylated DNA and a transcriptional repressor domain (TSD) that can inhibit transcription via the recruitment of histone deacetylases including mSIN3a, HDAC1 and HDAC2 [91].

MBD1, MBD2, MBD3 induced also repressed gene transcription through their interaction with proteins involved in chromatin remodelling [90]. In particular, MBD2 has been found to be associated with the NuRD/Mi-2 histone deacetylase complex and all together to form the MeCP1 complex. MBD3 is an internal component of the NuRD complex [92].

MBD4 is a DNA repair protein that repairs C→T transitions at CpG sites due to deamination of 5-methylcytosine and recognises methylated CpGs along with G/T mismatches [93]. Interestingly, many C→T transitions at CpG sites occurred at the MBD4 knockout mice.

In addition of repressing gene transcription, DNA methylation is associated with the inactivation of repetitive sequences, of X chromosome and genomic imprinting.

5.2.2 Repetitive sequences [adapted from Jörg Tost Epigenetics book ref (84)]

Repetitive sequences comprise a group of transposable elements or simple repeat sequences such as DNA satellites. The major human DNA satellites are the centromeric Sata, Sat2 and Sat3. Hypomethylation of these satellite repeats have been shown in the ICF syndrome as mentioned above. Transposable elements are DNA sequences that have the ability to integrate randomly into the human genome. Three types of transposable elements exist in the human genome: DNA transposons, the retrotransposons and endogenous retroviruses. DNA transposons are small elements that have a single transposase enzyme which is not functional due to a frameshift mutation. However, some remain active and favor the genomic instability. Retrotransposons are classified into those with a long terminal repeat in their sequence called LTR retrotransposons and those without called non-LTR retrotransposons [94]. These elements can move and integrate into other parts of human genome. When migrating into the promoters of the gene, they can modify gene transcription. LINE-1 elements, which are normally silenced by DNA methylation, belong to the group of non LTR retrotransposons. Their full length is 4-6kb and cover 21% of the human genome. Another group of transposons that depend on LINE-1 for transposition is the SINE family. The Alu repeats are also a member of this family. They are short in length (100-300bp) and cover 11% of the human genome.

5.2.3 X inactivation

X inactivation involves silencing of genes in one of two X chromosomes in females. The inactivation process involves the expression of untranslated transcripts such as Xist, which causes the heterochromatinization of X chromosome. The inactive state of the X chromosome depends on DNA methylation [95]. Different CpG sites along the chromosome are methylated and keep the silent state of gene expression in the X chromosome.

Imprinted genes are inactivated in one allele in a specific parental way. The regulatory elements of imprinted genes contain CpG islands that are methylated depending on the

parental origin. These regions are called imprinted control regions (ICR). The initiation of imprinting is performed during gametogenesis by DNMT3A/B. At this stage, DNA methylation marks are imprinted in a germ line specific manner. After fertilisation, the new imprinted code is propagated during development in somatic cells. An example of an imprinted locus, it is the insulin growth factor 2 (IGF2)/H19 locus [96]. These two genes are separated 70 bp and have the same transcriptional orientation. A specific ICR region is located between these two genes which regulate the expression of genes in a parental depending way.

5.3 Histone modifications

Another mechanism imprinted on the chromatin is the modification of histones (by acetylation, methylation, phosphorylation and ubiquitination) [97]. There are two classes of histones conserved in humans: the core histones H2A, H2B, H3, and H4 and the linker histones H1 and H5. Two of each of the core histones forme an octamer structure called the nucleosome. The DNA is wrapped around the nucleosome and forms a stable molecular structure. Post-translational modifications of the histone tails regulate the accessibility of transcription factors. These modifications, mainly located on lysine and arginine residues, are called “marks” and together determine the histone code. Histone acetylation and methylation are catalysed by the enzymes histone acetyltransferases and histone methyltransferases[98]. Molecular analysis of these marks showed that acetylation of H3/H4 lysines is associated with transcriptional activation, whereas methylation of H3 lysine 9 is associated with gene inactivation [98]. Hypoacetylation of H3 and H4 has been found in CD4 T cells from SLE patients and splenocytes from MRL/lpr lupus mice [99, 100]. Histones are also affected by citrullination. PADI4 is the enzyme that converts the methylated arginine to into citrulline. RA patients have autoantibodies against citrullinated antigen [101]. Whether or not histone citrullination is significant in the generation of autoantibodies, should be the subject of future investigation.

Histone deacetylases (HDACs) are the enzymes that remove the acetyl mark from the histone tails. Then, the chromatin changes conformation and the binding of transcription factors are inhibited. These enzymes interact with the DNA methylation proteins and together regulate gene transcription in somatic cells. HDACs have been reported to be deregulated in cancers as

well as in autoimmune diseases [102]. The first published report that the HDAC activity was found to be decreased in RASF synovium, suggested that inhibiton of HDACs is not necessary [103]. However, in several animal models of arthritis, HDACs inhibitors showed beneficial effects [104, 105].

6. Investigating DNA methylation in rheumatoid arthritis [adapted from Karouzakis E. et al. European Musculoskeletal Review 2008, 3: 41-43]

The area of research that studies epigenetic modifications regulating gene expression using different techniques is called epigenomics. Global changes of DNA methylation were studied in our group using two different methods. Immunohistochemistry is a useful technique to detect the expression of different proteins in patient biopsies. We used a modified immunohistochemistry protocol and mouse monoclonal antibodies directed against 5-methylcytosine to detect changes in DNA methylation between healthy individuals and patients with rheumatoid arthritis [106]. The same antibodies can also be used for fluorescence staining of cell nuclei. Fluorescence activated cell sorting (FACS) analysis can quantify the amount of 5-methylcytosine in the nuclei (Figure 9A). Another accurate quantification of 5-methylcytosine is high-performance liquid chromatography (HPLC) [107]. Here, the DNA is digested into single nucleotides and quantified by HPLC.

More specific approaches are used to identify gene targets that have been differentially methylated (Figure 9A). The bisulfite sequencing method can detect and quantify changes in CpG sites of gene promoters [108]. The assay is based on the bisulfite conversion of unmethylated cytosine to uracil, whereas 5-methylcytosine remains unchanged. After bisulfite treatment both unmodified and modified samples are amplified by PCR using specific bisulfite primers that are specific for a given gene locus. During PCR, uracils are converted into thymidines. Then, the PCR products are cloned into a plasmid vector and sequenced. Unchanged cytosine in the DNA sequence indicates the presence of a methylated cytosine, whereas a conversion from cytosine to thymidine indicates an unmethylated cytosine.

In combination with the above method, the addition of the pharmaceutical demethylating agent 5-azacytidine (5-azaC) can detect genes regulated by DNA methylation followed by RT-PCR or human genomic expression arrays (Figure 9B).

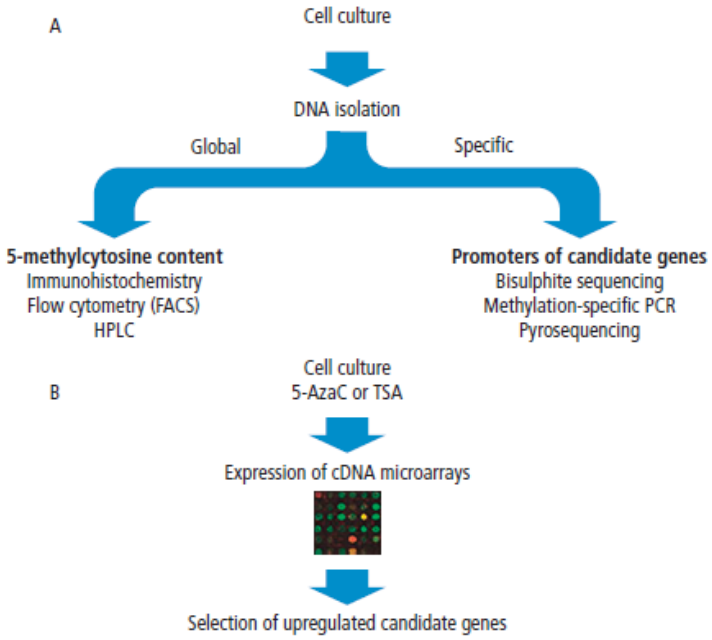

Figure 9: Methods to study DNA methylation changes in tissues and cell cultures. A) Global and specific analysis of DNA methylation marks. B) DNA hypomethylation and screening by complementary (cDNA) microarrays [adapted from Karouzakis E. et al. European Musculoskeletal Review 2008, 3: 41-43].

7. Objective

The major focus of the thesis is to understand the "spontaneous" activated phenotype of synovial fibroblasts from RA patients. To date, there are no genetic markers available to explain the invasive ability of RA synovial fibroblasts. Therefore, epigenetic analysis of RASF might provide important clues into the RASF activated phenotype. DNA methylation is an important modification that can lead to cellular transformation and differentiation. The aims of the current study are the following:

- To study global methylation in RA synovium and compare it with the OA synovium.
- To analyse the global methylation in RASF cell cultures and compare it with OASF cell cultures.
- To study the effect of pro-inflammatory cytokines on global methylation.
- To measure the expression of DNMTs in the synovium and cell cultures in order to find an explanation for the global DNA hypomethylation.
- To search for changes in gene and protein expression after genomic hypomethylation, with the hypothesis that demethylated fibroblasts from healthy individuals will acquire an activated RASF-like phenotype.
- To identify specific genes which are influenced by DNA hypomethylation and are important in the pathogenesis, and thereby describing new epigenetic markers in RA.

The information will improve our understanding of the activated phenotype of RASF and possibly provide new therapeutic strategies to reverse the destructive behaviour of those cells. In this study, we compared two groups of patients, suffering either from rheumatoid arthritis or osteoarthritis. The osteoarthritis synovial tissues and cell cultures were regarded as normal control cells, since they had no destructive properties. In chapter 2, we measured the global methylation in RA and OA synovial tissues, as well as in corresponding cultured synovial fibroblasts. For the demethylation and microarray analysis, we used a culture of normal synovial fibroblast obtained from the biopsy of a trauma patient. We investigated the possible result of DNA hypomethylation on those normal fibroblasts and performed cDNA microarray studies to analyse the changes in gene expression. Indeed, these cells acquired many characteristics of RASFs, including increased expression of cytokine receptors, adhesion molecules and matrix-degrading enzymes. Finally, in chapter 3, we analysed the promoter methylation of the chemokine CXCL12, which has been repeatedly found to be a differentially expressed gene between normal / osteoarthritis and RA synovial tissues.

Accordingly, its transcription and protein expression were upregulated in cultures of RASF *in vitro*. We hypothesized that the level of CXCL12 transcription is regulated by the methylation of its promoter. Indeed, we reported here CXCL12 as an epigenetic marker in RA.

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Chapter 2: DNA hypomethylation in rheumatoid arthritis synovial fibroblasts

Karouzakis E., Gay R.E., Gay S. Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. Arthritis & Rheumatism 2009; 60(12):3613-22.

1. Abstract

Rheumatoid arthritis synovial fibroblasts (RASF) are phenotypically activated and aggressive. We undertook this study to investigate whether the intrinsic activation of RASF is due to global genomic hypomethylation, an epigenetic modification.

Global genomic hypomethylation was assessed by immunohistochemistry, flow cytometry and LINE-1 promoter bisulfite sequencing. The levels of DNA-methyltransferase 1 (DNMT1) were determined in synovial tissues and cultured synovial fibroblasts by Western blotting, before and after treatment with cytokines and growth factors. Normal synovial fibroblasts (NSF) were treated for 3 months with a nontoxic dose of the DNA hypomethylation drug 5-azacytidine (5-azaC) and changes in gene expression were revealed using cDNA arrays. The phenotypic changes were confirmed by flow cytometry.

In situ and in vitro, RASF DNA had fewer 5-methylcytosine and methylated CG sites upstream of an LINE-1 open reading frame than did DNA of osteoarthritis synovial fibroblasts, and proliferating RASF were deficient in DNMT1. Using 5-azaC, we reproduced the activated phenotype of RASF in NSF. One hundred eighty six genes were upregulated more than two-fold by hypomethylation, with enhanced protein expression. These included growth factors and receptors, extracellular matrix proteins, adhesion molecules, and matrix-degrading enzymes. The hypomethylating milieu induced irreversible phenotypic changes in NSF, which resembled those of the activated phenotype of RASF.

DNA hypomethylation contributes to the chronicity of rheumatoid arthritis and could be responsible for the limitation of current therapies.

2. Introduction

Rheumatoid arthritis (RA), which affects approximately 1% of the population, is a chronic autoimmune disease involving progressive destruction of the affected joints. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) are involved in its pathogenesis [1-3]. Anti-TNFα therapies in particular have been shown to provide substantial benefit to patients not only through the reduction of signs and symptoms of the disease, but also by the inhibition of joint destruction [1]. However, treatments with current biologics, including anti-TNFα, anti-T cells and anti-B cells therapies, are only successful in at best in 60% of the treated patients and are still unable to cure the disease. A cytokine-independent pathway appears responsible for the ongoing joint destruction mediated by synovial fibroblasts (SF) [2]. Since implantation of rheumatoid arthritis SF (RASF) with human cartilage into SCID mice causes invasion into the cartilage without the support of the cells of the human immune system, it has been proposed that the activated phenotype is an "intrinsic" property of these cells [3].

SF, more than other types of fibroblasts, acquire phenotypic characteristics commonly associated with transformed cells [4]. RASF show "spontaneous" activities, associated with aggressive behavior and they differ from the SF of patients with osteoarthritis (OASF) or normal SF (NSF). For example, RASF up-regulate proto-oncogenes [5], specific matrix degrading enzymes [6], adhesion molecules [7], and cytokines [8]. These observations of an intrinsically activated cellular phenotype prompted us to search for epigenetic modifications.

In somatic cells, DNMT1 is the predominant DNA methyltransferase [9, 10]. Reduction of DNMT1 levels leads to hypomethylation, genomic instability, and tumorigenesis. Direct interaction between DNMT1 and proliferating cell nuclear antigen (PCNA) ensures that patterns of methylation are faithfully preserved in DNA synthesis [10]. Moreover, repetitive sequences such as LINE-1, Alu, and satellite alpha repeats are silenced by methylation in normal cells, and can be used as markers of global hypomethylation [11]. Our group and others [12, 13] reported a reactivation of the endogenous retroviral element LINE-1 in the RA synovial lining, and at sites of invasion. These reports suggest that global genomic hypomethylation plays a role in the pathogenesis of RA, and that genes normally silenced by methylation might contribute to the activated phenotype of RASF [12].

Here we show that DNA demethylation of normal synovial fibroblasts (NSF) induces a cellular phenotype resembling that of activated RASF. Genomic hypomethylation is a characteristic of RASF and is involved in the pathogenesis of RA.

3. Material and Methods

3.1 Cell cultures: RASF and OASF were isolated from synovial tissues obtained during joint replacement surgery. Normal SF were isolated from a small joint biopsy of a trauma patient. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal calf serum (FCS) and used between passages 5 and 6 (reagents were from Life Technologies, Basel, Switzerland). The procedure was approved by the Ethics Committee of the University Hospital, Zurich, Switzerland). The characteristics of the study patients were shown in Table 1.

	RA patients n=13	OA patients n=13
Age, mean (range) years	64 (48-79)	66 (54-88)
No. of women/no.of men	9/4	7/6
Medications, no.taking/no.assessed		
NSAIDs	2/13	2/13
DMARDs	9/13	0/13
Plus Steroid	4/13	0/13
Plus anti-TNF	2/13	0/13
RF,no. Positive/no.assessed *	6/13	NA
CRP,mean (range) mg/liter	35.9 (8.2-60.8)	2.4 (0.2-8.3)

Table 1. Characteristics of the study patients. RA= rheumatoid arthritis; OA = osteoarthritis; NSAIDs = non-steroidal anti-inflammatory drugs; DMARDs = disease-modifying antirheumatic drugs; anti-TNF = anti-tumor necrosis factor; NA= not assessed; CRP = C-reactive protein. * Rheumatoid factor (RF) positivity was defined as > 20 IU/ml.

3.2 Immunohistochemistry for 5-methylcytosine: Formalin fixed, paraffin-embedded sections of synovial tissues were deparaffinised and treated at 80°C for 30 minutes with citrate buffer (pH 3.4). The tissue slides were incubated with 2N HCL for 2 hours at 37 C. After acid treatment, the slides were washed well with Phosphate Buffer Saline (PBS) / 0.05% Tween-20 (PBST). To determine the methylation in the synovial tissues, mouse monoclonal antibodies against 5-methylcytosine (5-MeC) (Imgenex, San Diego, CA, USA) were used. Mouse IgG isotype (Dako, Glostrup, Denmark) served as negative controls. Double staining with vimentin (using murine anti-human monoclonal antibodies, clone V9, Dako) was used to

stain synovial fibroblasts. The antibodies were incubated overnight at 4°C. Bound antibodies were detected by incubation with biotinylated goat anti-mouse IgG (Jackon Lab., Bar Arbor, Maine, USA) for 1 hour. The slides were incubated with ABC reagents for HRP (Vectastain, Vector Lab., Burlingame, CA, USA).The methylcytosine modification and vimentin were visualised using 3.3-diaminobenzidine (Vector) and HistoGreen (Histoprime, Linaris, Wertheim-Bettingen, Germany) respectively. ImageJ software (National Institute of Health) was used to analyse the mean intensity of nuclei in the patient tissues. We acquired three different microscopic field images per patient tissue sample. The color images were converted into 8 bit gray/white images. The threshold was adjusted for each image in order to specify only the nuclei. The means of gray/white intensity of all nuclei in the image were obtained and compared. The numerical values given by software were converted to a mean intensity unit using the equation: Mean intensity = (1/mean of gray intensity /white intensity) x 100.

3.3 Flow cytometry for 5-MeC: The cells were fixed with 0.25% paraformaldehyde for 10 minutes at 37°C and kept on ice for 10 minutes before addition of 88% methanol / 12% PBS for 10 minutes at -20°C. The nuclei were washed twice with PBST-BSA and treated with 1N HCl for 40 minutes at 37°C. Neutralization of the acidic solution was performed by one wash step with 0.1 M borate buffer pH 8.5 and two wash steps with PBST/BSA. The nuclei were incubated for 20 minutes at 37°C with a blocking solution that contains PBST/BSA supplemented with 10 % FCS. The nuclei were incubated with anti-5-MeC antibodies (Imgenex) for 1 hour at 37°C, washed twice with PBS and incubated with anti-mouse FITC antibodies for 30 minutes at 37°C (BD Biosciences, Heidelberg, Germany). Finally, the samples were stained also with propidium iodine (PI) before being analysed using FACS (FACScalibur, BD Biosciences).

3.4 Bisulfite sequencing for LINE-1 promoter: Genomic DNA was prepared from RASF and OASF using the QiAmp DNA blood Mini kit (Qiagen, Hombrechtikon, Switzerland). The DNA (1μg) was bisulfite modified using the EpiTect bisulfite kit (Qiagen). The modified DNA was eluted in 20 μl of Tris Buffer (pH 8,5) and stored at -20°C. PCR amplification of bisulfite modified DNA (2 μl) was performed using Hot Start PCR and the AmpliTaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland). Primers were designed for the CpG area upstream of LINE-1 promoter (X58075, -420 to -49 bp) forward 5-TTT ATT AGG GAG TGT TAG ATA GTG GG-3 and reverse 5-AAA CCC TCT AAA CCA AAT ATA AAA TAT AAT-3. The online MethPrimer software was used

(http://www.urogene.org/methprimer/). The PCR purified fragment was cloned using the TOPO TA cloning kit according to manufacturer instructions (Invitrogen, Carlsbad, CA, USA). The positive clones were sequenced (Microsynth, Balgach, Switzerland). The data analysis was performed using the BiQ analyzer software (Max Plank Institut, Munich, Germany).

3.5 Immunohistochemistry for LINE-1 proteins: LINE-1 ORF1p and ORF2p were detected by immunohistochemistry on paraffin-embedded sections of synovial tissues. Rabbit polyclonal antibodies against LINE-1 ORF1p and chicken polyclonal antibodies against LINE-1 ORF2p (obtained from G.G. Schumann, Paul-Ehrlich-Institut, Section PR2 / Retroelements, Langen, Germany), anti-rabbit or anti-chicken biotinylated antibodies, streptavidin conjugated to alkaline phosphatase (Jackson Lab.) were used and Fast Red Substrate (Vector Lab.) revealed the staining. Non-immune rabbit or chicken sera were used as a control for primary antibodies.

3.6 Western blotting for DNMT1 and PCNA: Tissue or cells were prepared by lyses in RIPA buffer (50 mM Tris HCL pH 8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, reagents from Sigma-Aldrich, Buchs, Switzerland). Proteins were separated in 10% SDS-polycrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% nonfat dry milk with 0.05% Tween-20 in TBS (pH 7.4) and were incubated overnight with antibodies against human DNMT1 (Abcam, Cambridge, United Kingdom) or PCNA (PC10, Imgenex). After incubation with goat HRP-conjugated anti-mouse IgG secondary antibodies (Jackson Lab.) in 5 % non-fat dry milk with 0.05% Tween-20 in TBS (pH 7.4), bound antibodies were visualised using ECL chemiluminescence (Amersham, Buckinghamshire, United Kingdom). Intensity of the bands were evaluated by densitometry (Alphaimager 2200, Witec AG, Littau, Switzerland).

3.7 Treatment with TNFα, IL-1β or platelet derived growth factor (PDGF): RASF and OASF were passaged 48 hours and serum-starved (0,5% FCS) 24 hours before adding 10 ng/ml recombinant human TNFα, 1 ng/ml IL-1β or 10 ng/ml PDGF (R&D Systems, Minneapolis, MN, USA) (or medium alone) to the cell culture. The cells were kept in DMEM containing 10% FCS and collected after 24 or 48 hours.

3.8 Microarray of 5-Azacytidine induced gene expression: NSF (n=1) were treated with a low dose of 5-azacytidine (0,1 μM/ml 5-azaC for 3 months, with medium changed every 3 days over 2 passages) (Sigma-Aldrich) or left untreated (control group). The long incubation was used to have enough cell divisions and to mimic the chronic state of the disease. Total RNA was isolated with the RNeasy MiniPrep Kit (Qiagen) including treatment with RNase-free DNase. Double-stranded cDNA was synthesized from 5μg total RNA using the GeneChip One-cycle cDNA Synthesis Kit, including labeling with the One-cycle Target Labeling Assay (Invitrogen). The labeled cDNA was hybridized with the probe sets present on the Human Genome U133 Plus 2.0 Gene Expression Array (Affymetrix, Santa Clara, CA), using the Fluidics Station 450, according to standard protocols. The hybridization-picture was scanned with a GeneChip Scanner 3000 and further analyzed using the GCOS™ software. Data were normalized (measurements less than 0.01 were set to 0.01, and normalization per chip was set to 50th percentile) and analyzed with GeneSpring Microarray Analysis Software (Silicon Genetics, Redwood City, CA). Filters were set on 2-fold regulation, expression levels had to be >2.0, flags had to be present or marginal in at least one out of 2 compared samples.

3.9 FACS for phenotyping of RASF: RASF and OASF were treated with a low dose of 5-azaC (0,1 μM/ml 5-azaC for 2 weeks, with medium changed each 3 days). The cells were detached using Accutase (Omnilab) and incubated with the following primary antibodies or isotype controls for 1 hour at 4°C in DMEM including 10% FCS:anti-CD10,anti-CD26, anti-CD29, anti-CD36, anti-CD46, anti-CD130 (murine monoclonal antibodies, BD Pharmingen), anti- Matrix metalloproteinase 14 (MMP-14) hinge region (rabbit polyclonal antibodies, Chemicon, Zug, Switzerland), transforming growth factor β receptor 2 (TGFβ-R2), CK (murine polyclonal antibodies, Abnova, Heidelberg, Germany). Cathepsin K (CK) was measured in permeabilized cells (using DakoCytomation IntraStain). The cells were further incubated with fluorescein-conjugated secondary antibodies: goat anti-mouse IgG/IgM or rat anti-rabbit IgG (BD Pharmingen). The mean fluorescence intensity was determined by a FACScalibur.

3.10 Gene analysis: The following online programs were used: Ensembl genome browser (http://www.ensembl.org/index.html), CpGplot (http://www.ebi.ac.uk/emboss/cpgplot/), Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and TFBS list (http://lgsun.grc.nia.nih.gov/geneindex/mm6/TFBS/list.html).

3.11 Statistical analysis. Differences between patient groups were evaluated using the Mann-Witney U-test, whereas changes in the same cell culture were evaluated using the Wilcoxon signed ranks test. Analysis of frequency was performed using chi squared tests.

4. Results

4.1 Global genomic hypomethylation in RA synovial tissues. To visualize global genomic methylation, paraffin-embedded synovial tissue sections from RA and OA patients were stained with anti-5-MeC monoclonal antibodies (Fig. 1). In RA, the vimentin positive synovial fibroblasts showed decreased stainings of cell nuclei in both the lining and the sublining, reflecting a generalized genomic hypomethylation (Fig.1A). Detailed image analysis of OA and RA nuclei in the synovial lining and sublining showed that RA nuclei were stained less densely in comparison to OA nuclei (Fig.1B). Thus, synovial cell nuclei were significantly less methylated in RA, than in OA (Fig.1C, n = 6 each, $p < 0.05$).

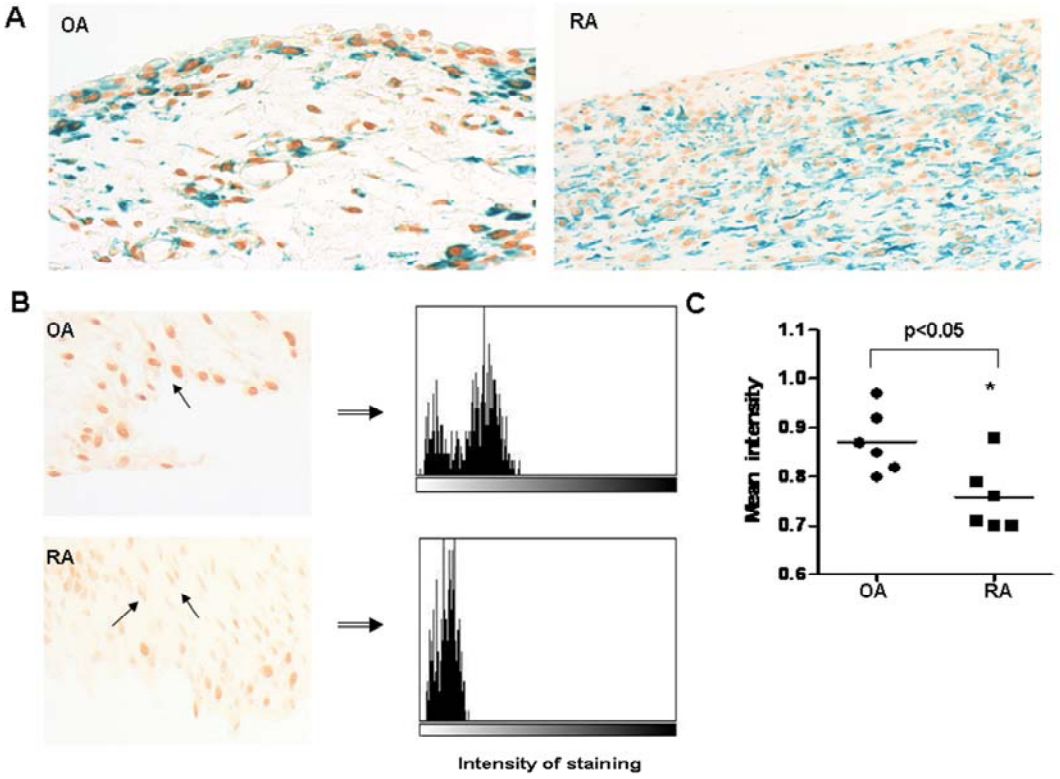


Figure 1. Immunohistochemistry shows global genomic hypomethylation in rheumatoid arthritis (RA) synovial tissue.

A) Osteoarthritis (OA) and RA synovial tissue was stained with monoclonal antibodies against 5-methylcytosine (brown) and vimentin (green). B) RA synovial fibroblasts showed lower intensity of brown staining (5-methylcytosine; arrows), than the OA synovial fibroblasts. Representative intensity histograms obtained with ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsbweb.nih.gov/ij/).**C,** RA synovial tissue was significantly less methylated than the OA synovial tissues, as reflected by

the lower nuclear staining of 5-methylcytosine (n = 6 samples each, determined by ImageJ software analysis). Horizontal bars indicate the mean. (Original magnification x 200 in A and B)

4.2 Global genomic hypomethylation in RASF and effects of cytokines and growth factors.

To determine whether RASF are still hypomethylated in vitro, SF were isolated from tissues and cultured for five to six passages. In addition, we investigated the effect on DNA methylation of exposure to physiological concentrations of pro-inflammatory cytokines and growth factors. Cells were stimulated or left untreated, and harvested. Cell nuclei were stained with anti-5-MeC monoclonal antibodies (Fig. 2A) and propidium iodide (Fig. 2B) and analyzed by flow cytometry. The cell nuclei of untreated RASF, compared with those of untreated OASF, showed significantly less 5-MeC staining (Fig. 2A/C, n = 6, $p<0.05$). In RASF, 5-MeC remained significantly reduced even in the presence of pro-inflammatory cytokines, TNFα or IL-1 (n = 6, $p<0.05$).

4.2.1 Cell cycle analysis.

We determined the percentage of OASF and RASF cells in the G2/M phase in the different conditions, i.e. with or without the addition of cytokines or growth factors (Figs. 2B/D). The strongest increase occurred with TNFα within 24 hours. However, no significant difference was detected between OASF and RASF in the different conditions (n = 6 each, p=0.8-0.9).

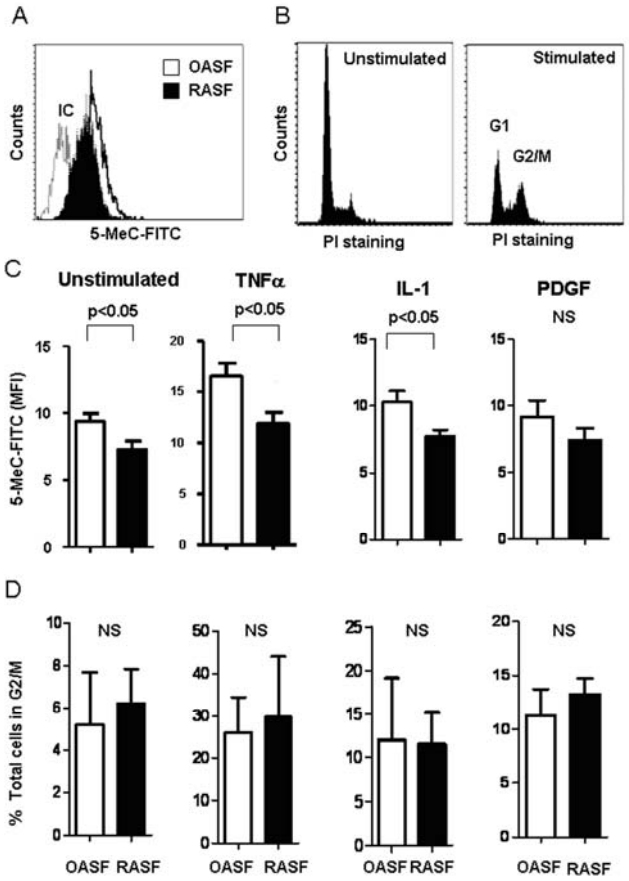


Figure 2. Flow cytometric analysis of cell nuclei confirms genomic hypomethylation of rheumatoid arthritis synovial fibroblasts (RASFs) in vitro; – effects of pro-inflammatory cytokines and growth factors. A) Nuclei of rheumatoid arthritis and osteoarthritis synovial fibroblasts (RASF and OASF) labeled with anti-5-methylcytosine (5-MeC)-FITC antibodies and evaluated by flow cytometry. Representative example of reduced 5-MeC in the nuclei of RASF (black), compared with OASF (white) (gray lines, IC: isotype control).

B) Example of cell cycle analysis using propidium iodine (PI) showing distinct G1, S and G2/M phases, in unstimulated synovial fibroblasts or upon exposure for 24h to TNFa.

C) Histograms of mean fluorescence intensity (MFI) of stainings with 5-MeC. Unstimulated RASF showed a significant reduction in 5-MeC. The cells were treated with TNFa, IL-1β or PDGF. In all conditions tested – except PDGF -, the relative deficiency of 5-MeC remains significant in RASF, when compared to OASF ($p<0.05$, $n = 6$ each).

D) Percentage of total cells in G2/M phase of the cell cycle in unstimulated and stimulated synovial fibroblasts (same experiment as in C). As expected, the proliferation increased upon stimulation, but no difference could be detected between OASFs and RASFs. NS = not significant. Values in **C** and **D** are the mean and SD.

4.3 Expression of DNMT1 and PCNA and effects of cytokines and growth factors.

We performed Western bloting with specific monoclonal antibodies to search for a defect in the methylation pathway. Tissue and cell lysates were analyzed for the expression of DNMT1 and PCNA. In RA synovial tissues, compared with OA synovial tissues, the ratio DNMT1/PCNA was significantly lower ($n = 6$ each, $p < 0.05$) (Fig. 3A). In RA, an increased rate of cell proliferation was associated with high levels of PCNA. In proliferating cells, it would be expected to find an increased expression of DNMT1; this was not the case in RA synovial tissues. The levels of DNMT1 were even lower in RA synovial tissue than in OA synovial tissue which showed low expression of PCNA and a low rate of proliferation. This observation suggested a deficient production or a decreased half-life of DNMT1 in the RA synovial tissue.

In vitro, the DNMT1/PCNA ratio remained significantly lower in RASF, than in OASF ($n = 6$, $p<0.05$) (Fig. 3B). We tested the effect of pro-inflammatory cytokines and growth factors on the expression of DNMT1 (Fig. 3C). Most importantly, in RASF, under all conditions tested, the DNMT1/PCNA ratio remained significantly lower, in comparison to OASF.

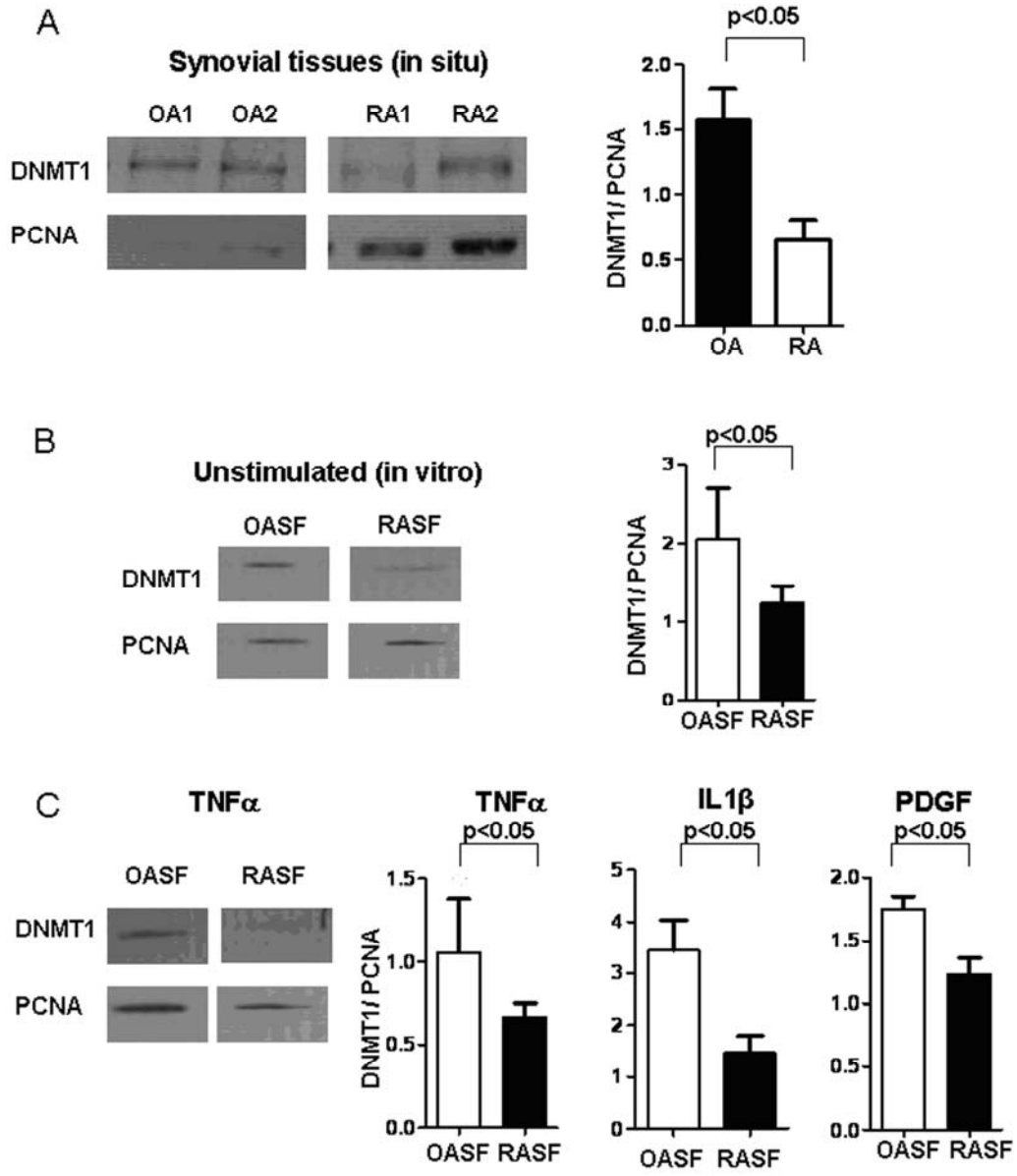


Figure 3. Relative deficiency of DNMT1 in proliferating cells.

A) The level of DNMT1 is very low in RA synovial tissue compared to OA synovial tissue, inspite of an increased expression of proliferating cell nuclear antigen (PCNA). Western blots show representative examples of DNMT1 and PCNA expression in synovial tissue from 2 OA patients and 2 RA patients. The PCNA/DNMT1 ratio was significantly reduced in RA (n=5 samples each).

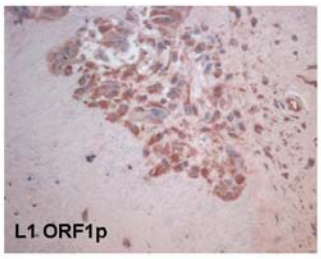
B) The expression of DNMT1 was decreased also in RA synovial fibroblasts (RASF) *in vitro*, compared to OA synovial fibroblasts (OASF) (left, representative examples). The histogram shows that the ratio of DNMT1/PCNA is significantly lower in RASF, than in OASF. (n = 6 samples each)

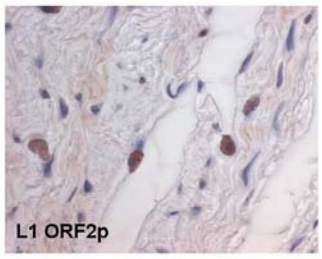
C) Representative Western blots show that stimulation of cells with TNFa for 24 hours increased the expression of PCNA, but the levels of DNMT1 in RASF remained very low. Importantly in all conditions tested (including those with IL-1b and PDGF), the DNMT1/PCNA ratio remained significantly reduced in RASF (n = 6 samples each). Values are the mean and SD.

4.4 Hypomethylated LINE-1 promoter in RASF.

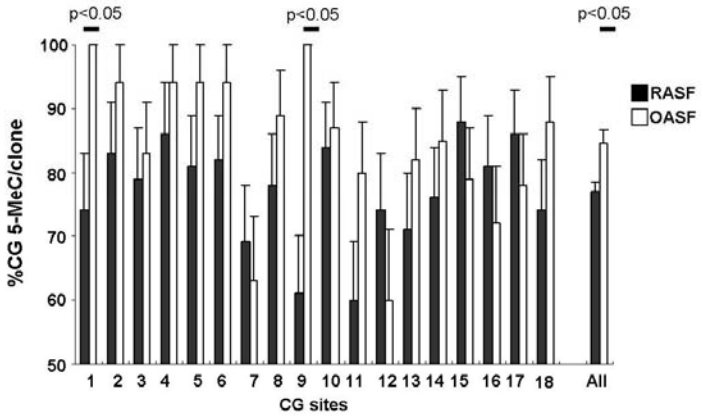
Because LINE-1 proteins are expressed in the RA synovial tissues (Fig. 4A), we thought to determine whether their expression is due to genomic hypomethylation. 18 CG sites of the LINE-1 promoter / 5'-UTR (i.e., GenBank-Nr. X58075, 372bp between nucleotides -49 and -420) were analyzed for changes in methylation by bisulfite sequencing. Genomic DNA derived from RASF revealed significantly fewer methylated CG sites upstream of the LINE-1 ORF1, in comparison with OASF (78 $\pm$ 2% versus 85 $\pm$ 3% CG methylation, $p < 0.05$, n = 7 patients, and 20 clones analyzed for each diagnosis) (Fig. 4B). Furthermore, we found that the methylation of L1 promoter in the isolated synovial fibroblasts correlated significantly with the expression of L1 ORF1p protein in the corresponding synovial tissue (Figure 4C).

A





B



C

	Unmethylated CpG	Missing CpG	Total	L1 ORF1(p40)
RA1	6/11 (54%)	7/18 (38%)	13/18 (72%)	+++
RA2	4/13 (31%)	6/18 (31%)	10/18 (55%)	+++
RA3	2/14 (14%)	5/18 (27%)	7/18 (38%)	++
RA4	2/18 (10%)	0/18 (0%)	2/18 (11%)	+
OA1	1/18 (5%)	0/18 (0%)	1/18 (5%)	+
r	0.89	0.95	0.95	
p	0.11	0.05	0.05	

Figure 4. LINE-1 proteins and promoter hypomethylation in RASF.

A) LINE-1 proteins (L1 ORF1p / p40 in fibroblasts at sites of cartilage destruction and L1 ORF2p / p150 in round-shaped cells at sites of bone destruction) are expressed in rheumatoid arthritis (RA) synovial tissues (original magnification x 400).

B) The LINE-1 promoter (5'-UTR, 372bp containing 18 CpG sites) is hypomethylated in RASF (black bars), compared with OASF (white bars). Bisulfite sequencing was performed in 25 clones from seven RASF and seven OASF cultures. The results were analyzed using BiQ-Analyser software and expressed in terms of mean plus standard deviation. CpG sites, particularly at positions one and nine, are significantly hypomethylated, in addition to a significant overall hypomethylation. The p values refer to differences between OASF and RASF (Mann-Whitney U-test). C) Correlation of LINE-1 promoter methylation in isolated synovial fibroblasts and L1 ORF1p protein expression in corresponding RA and OA synovial tissues. SF derived from RA tissue has a higher percentage of unmethylated and missing CpG than SF derived from OA tissue. The table shows the percentage of unmethylated CpG among the 18 CpG and the percentage of CpG which are missing from the patient genomic sequence versus the GenBank LINE-1 promoter sequence (Missing CpG). The total includes both

unmethylated and missing CpG. This has been correlated with the expression of L1 ORF1p (formerly called L1 p40 protein) using a semiquantitative evaluation of the immunohistochemical staining (from + = low expression to +++= high expression).

4.5 Phenotype of hypomethylated normal SF.

To explore whether a hypomethylating milieu is responsible for the activated phenotype of RASF, we continuously treated NSF (n=1) for three months with a non-toxic dose of the DNA hypomethylator 5-azaC, and analyzed the modification in gene expression using microarrays. 186 genes were more than two times upregulated during this condition. Many of these genes are implicated in RA, including interleukins, growth factors and their receptors, extracellular matrix proteins and enzymes, matrix degrading enzymes and inhibitors, adhesion molecules (Suppl. Table 1), protein kinases, transcription factors, components of Wnt, Ras and Rho-signaling pathways, and apoptosis-related proteins (Suppl. Table 2). The induced genes were categorized according to the occurrence of CpG islands in their respective promoters, in exons 1, or in both. Fifty-two of these genes had no CpG islands. One hundred and thirty-four of them have CpG islands in their promoters and/or exons 1 ($P < 0.001$). Transcription factors (18/22, $P < 0.005$) and adhesion molecules (10/11, $P < 0.01$) showed more CpG islands in their promoters and/or exons 1. We chose nine genes, which were >5-fold upregulated, and we measured their expression by flow cytometry in the presence and absence of 5-azaC.

4.6 Upregulation of genes with CpG islands in their promoters.

To confirm the 5-azaC expression microarray, OASF and RASF cultures (n=6, each) were treated for two weeks with a non-toxic dose of 5-azaC and the protein expression was analysed by FACS. Examples of genes with CpG islands in their promoters are CD10, CD29 and CD130. These gene products were significantly increased after two weeks of treatment with 5-azaC (Fig. 5A). Moreover, these proteins were significantly more expressed on the cell surface of untreated RASF than on OASF.

4.7 Upregulation of genes with CpG islands in exon 1.

Several genes that showed a > 5-fold upregulation of mRNA had no clear CpG island in their promoters. However, some of them had a CpG island in exon 1. Examples were CD26, matrix metalloproteinase-14 (MMP-14) and transforming growth factor receptor 2 (TGFβ-R2) (Fig. 5B). The baseline levels of CD26 and MMP-14 mRNA were the same in OASF and RASF. In presence of 5-azaC, however, CD26 and MMP-14 mRNA levels increased significantly over baseline in both OASF and RASF. TGFβ-R2 had a CpG island in exon 1, and its promoter showed an accumulation of CpGs. In OASF, 5-azaC increased the levels of expression significantly. However, in RASF, TGFβ-R2 expression on the cell surface was maximal in the absence and presence of 5-azaC.

4.8 Upregulation of genes without CpG islands in their promoters.

Several genes without CpG or increased frequency of CpGs up-regulate more than five-fold in the presence of 5-azaC, including CD36, CD46, cathepsin K (CK) (Fig. 5C). The basal expression of CD36 and CD46 on the cell surface was maximal in RASF and these values were significantly higher than those in OASF. Both are upregulated in OASF by 5-azaC. The baseline levels of CK were the same in OASF and RASF, but its expression was upregulated upon treatment with 5-azaC.

4.9 Differences between OASF and RASF in the response to hypomethylation.

In OASF**,** CD10, CD36 and CD46 showed significantly more increases in expression upon 5-azaC (Fig. 5D). This was in large part because their expression was already maximal in RASF.

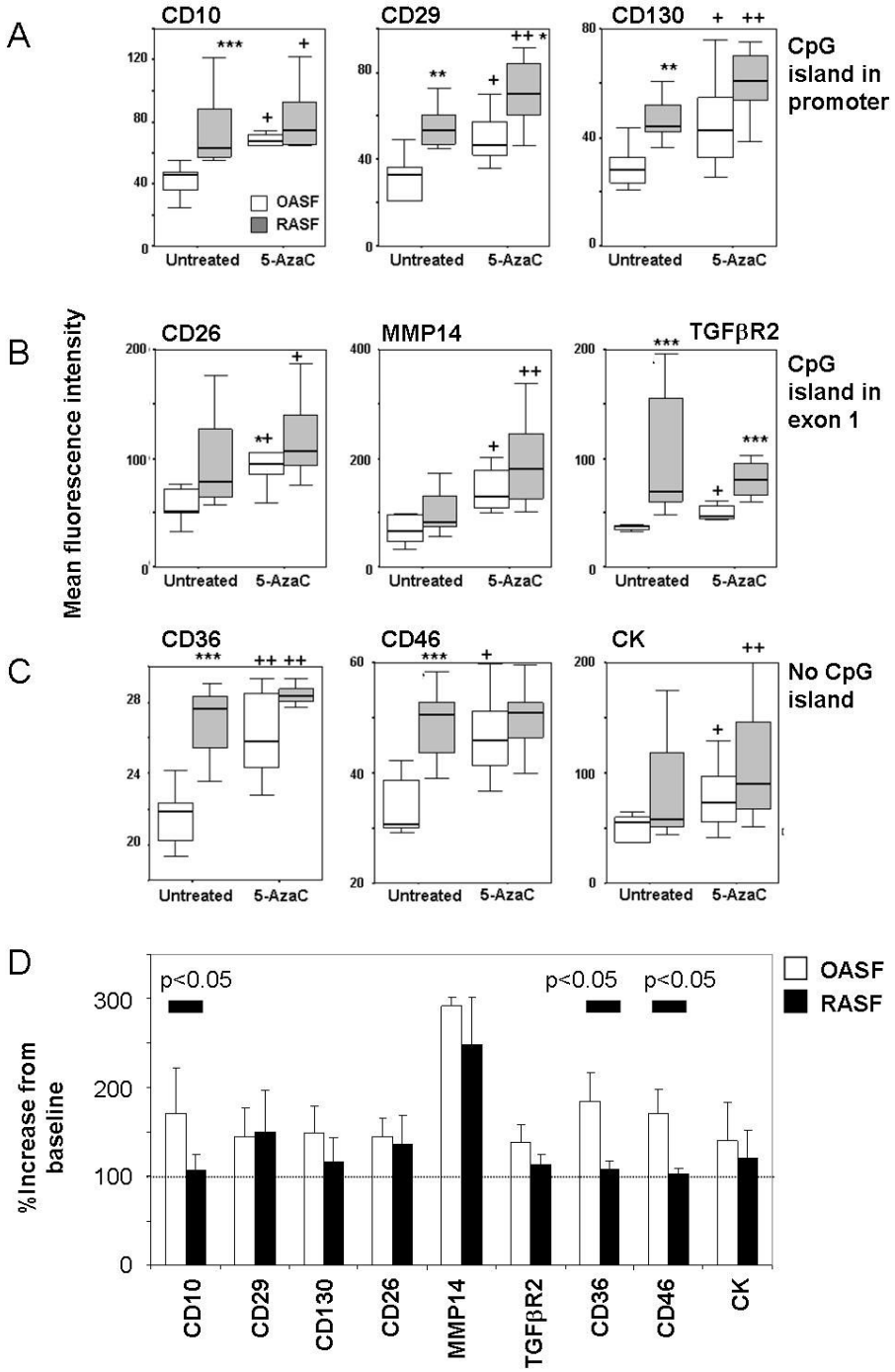


Figure 5. Protein expressions determined by flow cytometric analysis of gene products upregulated in a hypomethylating milieu in OASF and RASF.

A-C) Confirmation of the data obtained by Affimetrix cDNA arrays for normal synovial fibroblasts continuously treated with 5-AzaC, inducing genomic hypomethylation. Data are presented as box plots of mean fluorescence intensity, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th to 90th percentiles. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus

OASF, by Mann-Whitney U-test (n=6 samples per group) . + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, versus untreated control by Wilcoxon signed rank test.

D) Mean and SD percentages of increase from the baseline in gene expression in OASF and RASF. Significant differences in gene expression (n=6 patients to each diagnosis) between OASF and RASF in reaction to 5-AzaC treatment were observed for CD10, CD36 and CD46. MMP-14= matrix metalloproteinase 14; TGFβR2=transforming growth factor β receptor type II; CK=cathepsin K

5. Discussion

Here we report that genomic hypomethylation developed *in situ* is conserved in RASF *in vitro* even after >5 passages, and confirm that the expression of LINE-1 proteins in RASF is associated with a partially hypomethylated LINE-1 promoter. The degree of CG hypomethylation (78% methylation in RASF, versus 85% in OASF) in the LINE-1 promoter is similar to the degree of hypomethylation in tumor cells [9].

It is essential to understand more about this global genomic hypomethylation. Pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 have multiple influences on the pathogenesis of RA. IL-1β [14] and IL-6 [15] can affect genomic methylation. TNFα, however, had not previously been associated with epigenetic changes. We observed that a physiologic dose of TNFα accelerated the cell cycle, within 24 hours of exposure even more than IL-1β or PDGF. In OASF, stimulation of cell proliferation was accompanied by increased DNA methylation. This was not the case in RASF and therefore the relative degree of DNA hypomethylation remained in RASF treated with TNFα or IL-1β. As a consequence, it can be hypothesized that RASF become progressively more hypomethylated during inflammation. This results in further activation of genes in RASF.

Previous reports have shown that deficiency of DNMT1 is associated with genomic hypomethylation [10, 16, 17]. DNMT1 interacts with PCNA at the DNA replication fork, and this system is responsible for the transmission of methyl marks to daughter cells. In RASF, however, the expression of DNMT1 appeared deficient, either in unstimulated cells or after exposition to pro-inflammatory cytokines. Thus, in RASF, a relative deficiency of DNMT1 during cell proliferation could result in the observed genomic hypomethylation .

Our work raises the question of whether the global genomic hypomethylation is accompanied, or followed by, specific promoter hypermethylation, since this is the case in various tumors [9]. At least one example has been reported in the literature, i.e. by silencing the death receptor 3 [18], which could, at least in part, explain the relative resistance to apoptosis reported for RASF in certain patients [2].

In SF, the loss of methylation marks in daughter cells could cause an irreversible differentiation into an aggressive phenotype. Based on our observations, we hypothesized that NSF continuously treated with DNMTs-inhibitor 5-azaC will resemble RASF. Indeed, a large number of gene transcripts (73/186, 39%) found to be upregulated upon stimulation with 5-azaC and detected in cDNA microarrays, were previously described to be involved in the pathogenesis of RA. It is known that DNA methylation silence genes with CpG island promoters. Therefore, we choose three genes in the list provided by the microarrays, which showed > 5 fold five upregulation of mRNA and the presence of CpG islands in their promoters, namely CD10, CD29 and CD130. We confirmed that they were expressed on the cell surface of RASF more than on the surfaces of OASF, and that their expression was increased within two weeks of treatment with a low dose 5-azaC. It is well established that RASF attach to cartilage through adhesion molecules, including CD29 and CD61 (β1 and β3 integrins) [7, 19]. Invasion of RASF into cartilage requires the availability of these two integrins [19, 20]. CD10, a neutral endopeptidase, is highly expressed on RASF [21], and presumably plays a critical role in the local regulation of peptide levels in the joint. IL-6 signaling involves both a specific IL-6 receptor (IL-6Rα) and a ubiquitous signal-transducing protein, CD130 (gp130), which is also used by oncostatin M. Both IL-6 and oncostastin M are involved in the pathogenesis of RA [8, 22, 23].

We also evaluated the expression of genes that have no CpG island in their promoters, but do have a CpG island in exon 1, namely CD26, MMP-14 and TGFβ-R2. CD26 (dipeptidyl peptidase IV) was found to be highly expressed in RA synovial tissues [24] and in proliferating RASF [25]. The destruction of cartilage and bone in RA is in large part mediated by MMPs [20]. MMP-14 has a central role because it cleaves other pro-MMPs, and converts them into active forms. Inhibition of MMP-1 and/or MMP-14 results in a significant reduction of cartilage invasion by RASF [26]. Our results are in line with previous published data in pancreatic cancer cells showing that 5-azaC also upregulates the expression of MMP-14 and MMP-1 [27]. Expression of TGFβ-R2 was already maximal on RASF, as proposed earlier [28].

One fourth of genes (51/186, 27% in Suppl. Tables 1 and 2) that were upregulated upon treatment with 5-azaC in NSF contained no CpG island (e.g. CD36, CD46, CK, caspase-1 and IL-1R1). For example, CD36, abundantly expressed in RA synovial tissues, binds pro-

inflammatory oxidized low density lipoproteins [29] and thrombospondin-1 [30]. CD46 is a C3b- binding protein, which could be involved in tissue damage in RA [31], whereas CK, a key enzyme in bone resorption, is highly expressed in RA synovial tissue, not only by osteoclasts, but also by RASF [32]. Caspase-1 (IL-1β converting enzyme), which activates IL-1β in the RA synovial tissue [33], is also upregulated in NSF by 5-azaC. IL-1β stimulates both the synthesis and the activity of MMPs involved in cartilage destruction [34].

The proportion of gene products that were upregulated in NSF upon 5-azaC treatment, which are involved in intercellular processes and interactions with the extracellular matrix, and which have been described in RA synovial tissues and/or RASF was particularly high (52/73, 71% in Suppl. Table 1). These include twenty-two interleukins, growth factors and their receptors, thirteen extracellular matrix proteins and related enzymes; ten matrix-degrading enzymes and their inhibitors; and four adhesion molecules. Most importantly, among them, cathepsins, MMPs, mannosidase α1, carbonic anhydrases and ADAM12 are involved in joint destruction; and lysyl oxidase increased the crosslinking of mature collagen, an early step in cartilage destruction.

Many of the gene products also upregulated in NSF upon 5-azaC treatment are involved in intracellular processes, and play a role in RA (20/94, 21% in Suppl. Table 2). They include four protein kinases, ten transcription factors, two proteins in the Wnt pathway, two proteins involved in the regulation of actin filaments and Rho signaling, and two regulators of apoptosis. Furthermore, transcription factors whose expression increased upon 5-azaC (18/22, 82% in Suppl. Table 2) had CpG islands in their promoters and/or exon 1 more often than other genes revealed by the cDNA arrays. Many of them may play a role in RA, including Ets-related transcription factor, activating transcription factor 2 (ATF2; which binds to activator protein-1 (AP-1), C/EBPδ, NFAT5, CREB/ATF, HIF-2α and STAT1. The sustained upregulation of multiple signaling and transcription pathways in a hypomethylating milieu clearly could be responsible for the intrinsically activated phenotype of RASF. The cDNA arrays identified proteins that are implicated in the normal or pathological function of SF, including one recently reported as a potential autoantigen in RA, cartilage glycoprotein-39 (gp-39) [35].

CD10, CD36 and CD46 were also more upregulated in OASF than in RASF upon 5-azaC. The expression of all other genes tested was upregulated to the same extent in OASF and RASF. Bisulfite sequencing of the CD10 CpG island, however, showed that it is hypomethylated even in NSF (data not shown). Therefore, the gene is regulated indirectly or by a methylation-independent mechanism. Other investigators have reported similar effects of 5-azaC on myeloid leukemia genes [36]. CD46 and CD36 do not have a CpG island in their gene promoter, most likely they are regulated by indirect mechanisms. Other 5-azaC microarray studies have shown upregulation of genes in the absence of CpG islands in their promoters [37]. Thus, 5-azaC can apparently influence the expression of certain genes by different mechanisms. For example, it can affect histone modifications, up-regulate transcription factors, transcriptional repressors and/or the expression of microRNA[38, 39]. We have provided a list of transcription factors that could be candidates for future investigations (Suppl. Table 2).

In summary, we report reduced 5-MeC DNA in RA synovial tissues and in cultured RASF. Specifically, the promoter of an LINE-1 element was partially demethylated, confirming the global genomic hypomethylation in RASF. Moreover, our observations suggested a progressive loss of methylation marks. It can be hypothesized that: 1) the loss of methylation marks could be responsible for the intrinsically activated and aggressive phenotype of RASF and 2) that tissue-specific transcription factors, which are not normally expressed in synovial tissues, are upregulated in the disease and can be responsible for the activation of many genes involved in the pathogenesis of RA. Moreover, genomic hypomethylation could explain the increased expression of multiple receptors, adhesion molecules, and matrix-degrading enzymes, which play a role in RA and explain the enhanced response of RASF to pro-inflammatory cytokines, leading all together to joint destruction. Thus, the epigenetic modifications of RASF may be responsible, at least in part, for the fact that current therapies do not work in all patients and do not cure the disease yet.

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Supplementary information

Suppl. Table 1. Upregulated expression of genes involved in intercellular processes and interactions with the extracellular matrix (> 5-times open, > 2 times in parenthesis) in normal synovial fibroblasts cultured in a hypomethylating milieu, as revealed by Affimetrix cDNA arrays. The genes were grouped according to function and occurrence of CpG islands. Gene products previously associated with rheumatoid arthritis are marked with an * (PubMed).

	No CpG island	CpG island	CpG island exon 1	CpG island promoter/exon 1
Interleukins/growth factors and receptors	IL-1R1* IGF-1* PDGF-R-like* (Caspase-1)* (CCL4)* (CCRL1)* (CD105/Endoglin)* (FAPα)* (IL-12A)* (IL-13Rα2)* (PHRH)* (TGFβ-R3)*	CD130/gp130* (Activin A-RIIA)* (IL-13Rα1)* (Proenkephalin A)* (TGFβ-R1)*	TGFβ-R2* TFPI-2 Neuromedin B (Angiotensin II-R1)* (EGF-like EDIL3)	CD232/Plexin C1 (EGF-R v-erb-B)* (DCBLD2) (IFITM1) (Osteocalcin)* (Pentraxin 2) (Sortilin 1) (VEGF-A)*
ECM proteins and enzymes	CD36* CD46* gp-39* Laminin α4 Proteoglycan 4 (Asporin)* (Dermatopontin) (Sarcoglycan β) (Utrophin)	CD10* Fibronectin 1* (APLP2) (Bamacan) (USP10) (USP34)	CD26* (SCARB1) (Brevican) (Collagen VI α1)* (Lysyl oxidase)* (Thrombomodulin)*	(CD142/tissue factor F3)* (Fibronectin FLRT2) (Fibronectin FNDC3A) (Glypican 3)* (Mannose-binding lectin)[54] (Periplakin)
MMPs and inhibitors	Cathepsin K* MMP-1* (Cathepsin S)*	(MAN1A1)*	MMP-14* (Cathepsin L)* (TIMP-3)*	Carbonic anhydrase XII* Cathepsin H* GNS (ADAM12)* (Serine protease 12)
Adhesion molecules	(CLEC2B)	CD29/β1 integrin* (Protocadherin 9)	(Fibulin 1) (Protocadherin β12) (Protocadherin γA3) (Tetraspanin 6)*	Protocadherin γC3 (CD61/β3 integrin)* (β8 Integrin) (Tetraspanin 2)*
Others	Apolipoprotein E*			Cyclooxygenase 1* Glutathione peroxidase 3*

Suppl. Table 2. Upregulated expression of genes involved in intracellular processes (> 5-times open, > 2 times in parenthesis) in normal synovial fibroblasts cultured in a hypomethylating milieu.

	No CpG island	CpG island	CpG island exon 1	CpG island promoter/exon 1
GTPases and protein kinases	(Kynureininase) (TANK) (GIT2)	(PRKAR1A)* (PRKG1)* (TRAF3 IP2)	(TNIK)* (RIPK5) (MAPKKKK5) (MAPKK2)	(MAPK14 / p38a)* (MAPKKK8)
Transcription factors and regulators	TPR-MET* (ELF1)* (ODZ1) (RUNX1)*	(ATF2)* (BACH1) (BCLAF1) (MBTPS1) (TCFL2)	(AFF2) (C/EBPδ)* (NFAT5)* (SOX4) (STAT2)*	(CREBZF)* (GTF2I) (GTF3C2) (HIF2α)* (MBTPS2) (PHTF2) (SMAD2)* (STAT1)*
Wnt/Frizzled signalling	Wnt1 IP2		SFRP4*	(SFRP1)*
Actin filaments/ Rho signaling	Gelsolin* (ARHGAP1) (ARHGEF3) (Caldesmon 1) (PHACTR2) (Tenascin C)*		(KLHL24) (RHOBTB3) (MFAP3) (Myomegalin)	(ROCK2) (Podoplanin) (SMARDCD2)
Apoptosis		(Formin BP3) (RNF6) (TXNIP)* (WWOX)*	(MAGD4)	(API5) (PIP4K2B)

Others	ATP13A3	DHX9	EIF4EBP2	ASPHD1
	ATP2A1	PTPN12	(ARL4C)	CRISPLD2
	COPA	(CHP1)	(KCND2)	(ATP2A2)
	EVI2A	(FBOX38)	(RAB3B)	(ATP6V1C1)
	EVI2B	(FKNB15)	(SF3B1)	(CACNA2D1)
	SLC16A7	(PLD1)	(SLC16A2)	(Cyclin G2)
	(DBT)	(RAB27A)		(ENPP1)
	(RBM9)	(SEL1L)		(GNA13)
	(REP-1)	(STEAP1)		(PCM1)
	(SERPINF1)			(PTPN11)
	(SNAP23)			(ZKSCAN1)

Chapter 3: DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts

Karouzakis E., Rengel Y., Jüngel A., Kolling C., Gay R.E. , Michel B.A. , Tak P.P. , Gay S., Neidhart M., Ospelt C. DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. Genes & Immunity (2011) in press

1. Abstract

In the search for specific genes regulated by DNA methylation in RA, we investigated the expression of CXCL12 in SF and the methylation status of its promoter and determined its contribution to the expression of matrix metalloproteinases (MMPs).

DNA was isolated from SF and methylation was analysed by bisulfite sequencing and McrBC assay. CXCL12 protein was quantified by ELISA before and after treatment with 5-azacytidine. RASF were transfected with CXCR7-siRNA and stimulated with CXCL12. Expression of MMPs was analysed by Real-Time PCR.

Basal expression of CXCL12 was higher in RASF than osteoarthritis (OA) SF. 5-azacytidine demethylation increased the expression of CXCL12 and reduced the methylation of CpG nucleotides. A lower percentage of CpG methylation was found in the CXCL12 promoter of RASF compared to OASF. Overall, we observed a significant correlation in the mRNA expression and the CXCL12 promoter DNA methylation. Stimulation of RASF with CXCL12 increased the expression of MMPs. CXCR7 but not CXCR4 was expressed and functional in SF.

We show here that RASF produce more CXCL12 than OASF due to promoter methylation changes and that stimulation with CXCL12 activates MMPs via CXCR7 in SF. Thereby we describe an endogenously activated pathway in RASF which promotes joint destruction.

2. Introduction

A variety of studies describe an activated phenotype of synovial fibroblasts (SF) in rheumatoid arthritis (RA) which is characterized by a changed morphology, deranged apoptotic behaviour, and increased invasive properties [1]. Recently, we found that epigenetic changes might contribute to these phenotypic changes in RASF. We could show that DNA of RASF is globally hypomethylated when compared to osteoarthritis (OA) SF or normal SF and that less DNA methyltransferase DNMT1, the enzyme responsible for DNA de novo methylation is present in RASF [2]. DNA methylation is well known to regulate gene expression. Transcriptional regulation by DNA methylation occurs in CpG rich regions of gene promoters, so called CpG islands. Methyl CpG binding proteins bind to methylated CpG islands and together with chromatin remodelling enzymes cause gene silencing [3]. In the normal genome, DNA methylation regulates gene expression to form tissue specific expression patterns [4]. Aberrant DNA methylation was found in various pathologies including cancer and autoimmune diseases [5, 6].

CXCL12, also known as stromal-derived factor-1 (SDF-1) is a key player in the trafficking of lympho- and hematopoietic progenitor cells and in the early development and regeneration of tissues [7]. Moreover, the secretion of CXCL12 is increased after tissue damage by hypoxia, toxins or irradiation, leading to the immigration of progenitor cells expressing the CXCL12 receptor CXCR4 [8]. A second receptor for CXCL12, CXCR7 has only most recently been identified and has been implicated in tumor growth and metastasis [9-11]. Elevated levels of CXCL12 were found in patients with multiple sclerosis, inflammatory myopathies, spondyloarthropathies and RA [12-17]. Levels of CXCL12 in the synovial fluid of RA patients are around 10 times higher than in healthy joints and reach a mean of 750 ng/ml [16]. It is understood that CXCL12 drives chronic inflammation by attraction of monocytes and lymphocytes into the joint and by stimulation of synovial fibroblasts to produce pro-inflammatory cytokines [17-20]. Previous studies also showed that cultured RASF produce more CXCL12 than normal or OA synovial fibroblasts [16, 21].

Based on these results, we analyzed in the current work whether expression of CXCL12 in RASF is modified by changes in DNA methylation. Furthermore, to elucidate CXCL12 signaling pathways we looked at the expression of CXCR7 in synovial fibroblasts and tested whether CXCR7 mediates the production of matrix-metalloproteinases (MMPs) after CXCL12 stimulation in RASF.

2. Material and Methods

2.1 Patients and tissue preparation: Synovial tissues were obtained from trauma patients (normal synovial fibroblasts, NSF, n=2), RA n=14 and OA n=11 patients undergoing joint replacement surgery at the Schulthess Clinic Zurich after written consent (Table 1). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [22]. Normal lung fibroblasts were isolated from open lung biopsies of patients suspected with cancer but with a negative diagnosis. Synovial fibroblasts and normal lung fibroblasts were isolated and cultured as described previously [23].

2.2 Bisulfite sequencing. Genomic DNA was prepared from synovial fibroblasts using the QiAmp DNA blood Mini kit (Qiagen, Hombrechtikon, Switzerland). The DNA (1μg) was bisulfite modified using the EpiTect bisulfite kit (Qiagen). Two rounds hemi-nested PCR amplification of bisulfite modified DNA (2 μl) was performed using the AmpliTaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland). The PCR cell cycle program was 95°C 4 min ; 95°C 30s, 52°C 90 sec, 72°C 2 min x 5 ; 95°C 30sec, 52°C 90sec, 72°C 90 sec x 25 ; 72°C 4 min. Primers were designed for two regions of the CXCL12 CpG island promoter. The forward 5-GTT TGT GAT TAG TTT ATT TTA TTA-3, reverse 5-CTA AAT AAA AAC CAA TAA AAA AC-3 and hemi-reverse 5-AAA AAA TCC TAC TTT CTA TAC-3 bisulfite sequencing primers amplified the region -741bp to -477bp and the forward 5-GTT TTT TAT TGG TTT TTA TTT AGT TTT-3, reverse 5- TAC CTC CAC CCC CAC TAT AT-3 and hemi reverse 5- GAG TTT GAG AAG GTT AAA GGT-3 bisulfite sequencing primers amplified the region -244bp to +272bp. The computer software MethPrimer predicted CpG islands with the following criteria: island size >100, GC content > 50 % and Obs/Exp >0.6 and designed bisulfite primers for DNA methylation analysis [24]. The PCR purified fragments were cloned using the Qiagen PCR cloning handbook according to manufacturer instructions (Qiagen). Positive clones were sequenced (Microsynth, Balgach, Switzerland). The data was analysed using the BiQ analyzer software [25].

Table 1. Characteristics of the study patients

Diagnosis	Patient Number	Age (years)	Gender	Disease duration (years)	NSAIDs	DMARDs	RF pos (>20 I.U.)
RA	1	55	f	18	+	steroids MTX hydroxychloroquine	+
	2	64	f	9	-	steroids	+
	3	70	m	4	+	steroids	+
	4	65	f	15	-	MTX	+
	5	52	f	19	-	Actemra,MTX	+
	6	69	f	28	-	Humira	+
	7	45	f	17	-	Salazopyrin,Arava	+
	8	65	m	15	-	MTX	+
	9	52	f	21	-	Arava	NA
	10	66	f	46	-	MTX	+
	11	67	f	37	-	Mabthera	+
	12	66	f	16	-	Arava, Prednison	+
	13	73	f	7	-	Prednison	+
	14	79	f	30	-	Prednison	NA
OA	1	95	f	NA	-	-	NA
	2	70	f	NA	+	-	NA
	3	53	f	NA	+	-	NA
	4	61	f	NA	-	-	NA
	5	71	m	NA	-	-	NA
	6	73	f	NA	-	-	NA
	7	70	m	NA	-	-	NA
	8	82	f	NA	-	-	NA
	9	62	m	NA	-	-	NA
	10	79	m	NA	-	-	NA
	11	72	f	NA	-	-	NA

RA: rheumatoid arthritis; OA: osteoarthritis; f/m: female/male; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs; RF pos: rheumatoid factor positivity; MTX: methotrexate; NA : not assessed.

2.3 McrBC digestion and quantitative Real-time PCR. The CXCL12 promoter DNA methylation was determined by methylation-sensitive McrBC-PCR assay. Genomic DNA (1μg) was sonicated using the Diagenode bioruptor (10 min, 15 sec on and 15sec off). The sonicated DNA was digested with 10 units of McrBC or mock undigested in a 50μl reaction mixture containing 1x NEB2 buffer, 10mM GTP and 0.1mg/ml BSA. The restriction digestion reaction was incubated at 37 ^{o}C overnight and then the reaction was deactivated with heating at 65 ^{o}C for 20 min. The McrBC treated DNA and mock samples were purified using Qiagen PCR purification protocol kit. Quantitative SYBR green PCR using the CXCL12 promoter (-741 to -477) primers: Forward CAC CAT TGA GAG GTC GGA AG, Reverse AAT GAG ACC CGT CTT TGC AG, was carried out with the McrBC digested and mock samples. McrBC cleaves methylated DNA strands and inhibits PCR amplification. In contrast, unmethylated DNA prevents McrBC cleavage and can be measured by quantitative PCR. Mock undigested DNA is considered as total amount of DNA used in the reaction. Methylated DNA will have decreased Ct values after McrBC digestion. The Real-time PCR results were normalized with the mock treated sample DNA (deltaCt) and presented as fold induction of PCR recovery after digestion with McrBC.

2.4 Stimulations. RASF and OASF were seeded at low density 24h before treatment. RASF were stimulated with 100 ng/ml recombinant CXCL12 (Peprotech, London, UK). OASF were treated with 0.5 μM or 1 μM 5-azacytidine (Sigma) for 6 days. The cell culture medium was changed every 24h and replaced with new 5-azaC.

2.5 Transfection of CXCR7 small interfering RNA (siRNA). siRNA for CXCR7 and double stranded siRNA without homology to mammalian genes (negative control) (both from Qiagen) were used for silencing experiments. Also, transfection without siRNA (mock) was performed to determine whether the experimental set-up causes unspecific effects. The transfections were done by electroporation (Nucleofector, Lonza, Cologne, Germany) using transfection reagents for primary mammalian fibroblasts (Basic Nucleofector® Kit; Lonza) at a concentration of 0.9 μg siRNA/5x10^{5} cells. Transfected RASF were cultured for 48h before efficiency of knock-down was measured by Real-time PCR, FACS and Western blot or before stimulations were begun.

2.6 RNA isolation and quantitative Real-time PCR. Total RNA was isolated using the RNeasy Miniprep kit (Qiagen) including DNase treatment. RNA was reverse transcribed

using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). Samples without addition of reverse transcriptase served as negative control (non RT). Relative quantification of mRNA levels by TaqMan®/SYBRGreen® Real-time PCR was done using eukaryotic 18S ribosomal RNA as endogenous control (Applied Biosystems). The differences of the comparative threshold cycles (Ct) of sample and 18S cDNA were calculated (dCt). Relative expression levels were calculated following the formula ddCt = dCt (sample stimulated) - dCt (sample unstimulated). Relative expression was calculated using the expression 2^{-ddCt}. The sequence of the primers used for measuring MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, TIMP-1, TIMP-2, TIMP-3 were previously described [26].

2.7 PCR. mRNA was reverse transcribed using oligo dT and Moloney Murine Leukemia Virus reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). PCR was performed on a C1000™ Thermal Cycler (Bio-Rad laboratories, Hercules, CA) with the following primer pairs and protocols designed to detect the 4 different CXCR4 mRNAs as published on the National Center for Biotechnology Information AceView database. CXCR4 transcript 1: forward primer GGAAAAGATGGGGAGGAGAG, reverse primer CACTTCCAATTCAGCAAGCA; CXCR4 transcript 2: forward primer CAGCAGGTAGCAAAGTGACG, reverse primer GTAGATGGTGGGCAGGAAGA; CXCR4 transcript 3: forward primer AAGGGTCACCGAAAGGAGTT, reverse primer GAAGAGACCGGTGGTCTGAG; CXCR4 transcript 4: forward primer GTTAAGCGCCTGGTGACTGT, reverse primer GGTAACCCATGACCAGGATG. 94°C 5min, 40 cycles with 94°C 30s, 58°C 30s, 72°C 30s and a final elongation of 5min with 72°C. β-microglobulin: forward primer AAGATTCAGGTTTACTCACGTC, reverse primer TGATGCTGCTTACATGTCTCG. 94°C 5min, 35 cycles with 94°C 30s, 55°C 30s, 72°C 30s, final elongation 72°C 5min. Reaction products were separated on a 1.5% agarose gel and signals were visualized using ethidium bromide.

2.8 Western blot analysis. Whole cell lysates were prepared by lysing cells in 2x Laemmli buffer. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). Membranes were blocked for 1h at room temperature in 5% nonfat dry milk with 0.05% Tween 20 in TBS and were incubated overnight with polyclonal rabbit anti-human CXCR7 (2.8 μg/ml; Abcam, Cambridge, UK). To ensure specificity of the CXCR7 antibodies, blots were incubated with

CXCR7 antibodies which were pre-incubated for 1h at 37°C with or without 10 μg/ml of a synthetic CXCR7 peptide (10 μg/ml; Abcam). Afterwards, membranes were incubated with HRP-conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK). Bound antibodies were visualized using enhanced chemiluminescence (Amersham Bioscience, Otelfingen, Switzerland). Equal protein loading was confirmed using mouse anti-human α-tubulin antibodies or Ponceau solution (Sigma-Aldrich, Basel, Switzerland).

2.9 Flow Cytometry. RASF were detached from the culture flask with accutase (PAA Laboratories, Linz, Austria), washed with PBS/1%FCS and incubated with 20 μg/ml of rabbit anti-human CXCR7 (Abcam, Cambrige, UK) or of rabbit IgG for 45 minutes at 4°C. Cells were washed with PBS/1%FCS and subsequently incubated with FITC-labeled goat anti-rabbit IgG for 45 minutes at 4°C. Washing steps were repeated twice before analysis of the cells in the flow cytometer (FACSCalibur). Data were processed using CellQuest software (BD Biosciences, San Diego, CA).

2.10 ELISA. Synovial fibroblasts were seeded at 1 x 10^5/ml DMEM and CXCL12 was measured after 24h (Figure 1B) or at 2 x 10^5/ml DMEM and CXCL12 was measured after 24h and 96h (Figure 5C) in the supernatants with the DuoSet® ELISA development system (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

2.11 CXCL12 blockade. RASFs (2 x 10^5) were incubated with 1 μg/ml neutralizing anti-human CXCL12 antibodies (R&D Systems) or IgG control. Fresh antibodies or control was added after 48h.Cells were lysed after 24h or 96h.

2.12 Statistical analysis. All data are expressed as mean ±SEM. Statistical analysis was performed using GraphPad Prism software, version 4.03 (GraphPad System, San Diego, CA). For analysis between different groups, the Mann-Whitney U, for paired analysis Wilcoxon signed rank test was used. *P* values less than 0.05 were considered significant.

3. Results

3.1 RASF upregulate CXCL12 mRNA and protein expression.

To analyse the basal expression of CXCL12 expression in RASF and in OASF, we measured CXCL12 mRNA and protein levels in cell culture supernatants after 24h. RASF express significantly more CXCL12 mRNA than OASF and NSF (RASF 6.9±0.3 dCt, n=11; OASF 8.3±0.5 dCt, n=8; NSF 7.5 dCt, n=1; $p<0.028$) (Fig 1A). Furthermore, we observed that the mRNA expression of CXCL12 was positively correlated with the C-reactive protein, a marker of inflammation marker in these patients ($r = -0.64$, $p<0.01$). RASF produced with 85±12 pg/ml also significantly more CXCL12 protein than OASF with 52 ±10 pg/ml (Fig.1B).

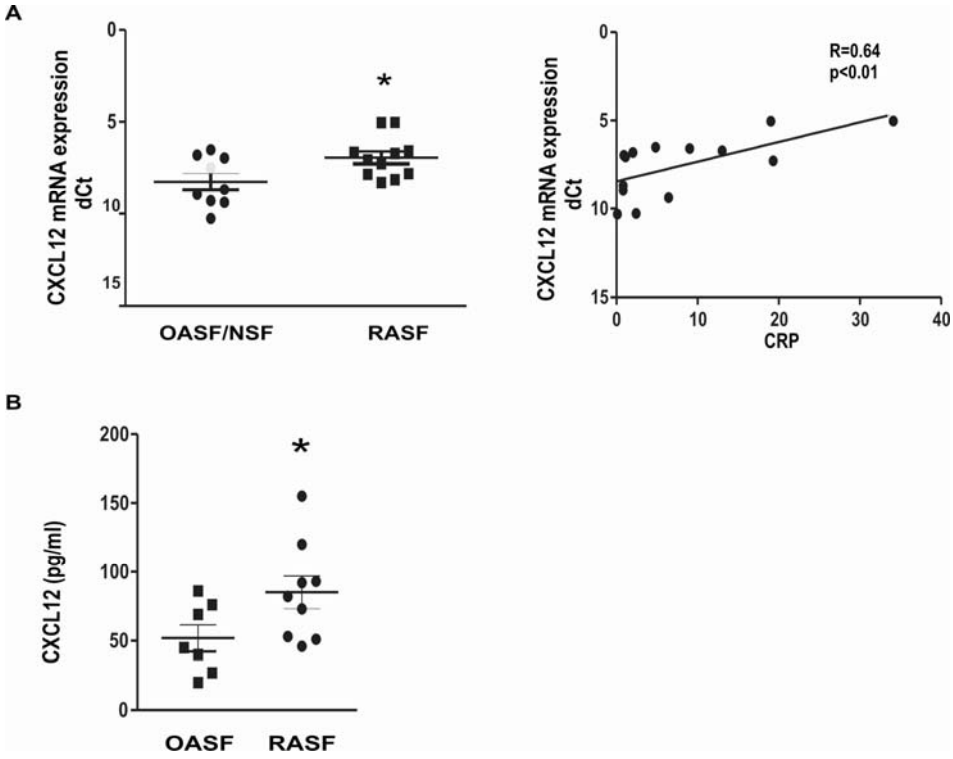

Figure 1. RASF express more CXCL12 mRNA and secrete more protein than OASF. A) RASF (n=11, ■) expressed significantly more CXCL12 mRNA than OASF (n=8, ●) and NSF (n=1, ●). The mRNA expression of CXCL12 correlated with the patients CRP levels. B) ELISA was used to analyse the amount of CXCL12 release in RASF (n=9) and OASF (n=7) cell culture supernatants. RASF released significantly more CXCL12 than OASF. * $p<0.05$

3.2 DNA methylation regulates the promoter of CXCL12

To investigate whether changes in DNA methylation might influence expression of CXCL12 in RASF, we first tested whether the CXCL12 promoter can be regulated by DNA methylation. Computational analysis predicted that there are CpG islands upstream of the transcription initiation site of the CXCL12 promoter (Fig. 2A). We further analysed the region that is 100 bp upstream of exon 1 (-741bp to -477bp) and the region that spans exon 1 (-244bp to +272bp) by bisulfite sequencing. Our findings showed that NSF have a high percentage of methylated CpG nucleotides in the promoter upstream region from -741bp to -477bp, while in the region -244 to +272, that spans the transcriptional initiation site and exon 1, all the CpG nucleotides were unmethylated (Fig. 2B). To see whether demethylation of the heavily methylated CpG rich region would influence CXCL12 expression, we treated OASF with different doses of the demethylating drug 5-azaC and found dose dependent upregulation of CXCL12 protein secretion (Fig. 2C). Furthermore, bisulfite sequencing analysis showed demethylation of the CXCL12 promoter (-741bp to -477bp) with 1 μM 5-azaC (Fig. 2D). In addition, the mRNA expression of CXCL12 correlated with the methylation levels of the CXCL12 promoter ($r = 0.60$, $p < 0.04$) as show my McrBC digestion assay (Fig. 2E).Taken together, the results of figure 2 indicate that expression of CXCL12 is regulated by methylation of its promoter, and accordingly can be increased by demethylation.

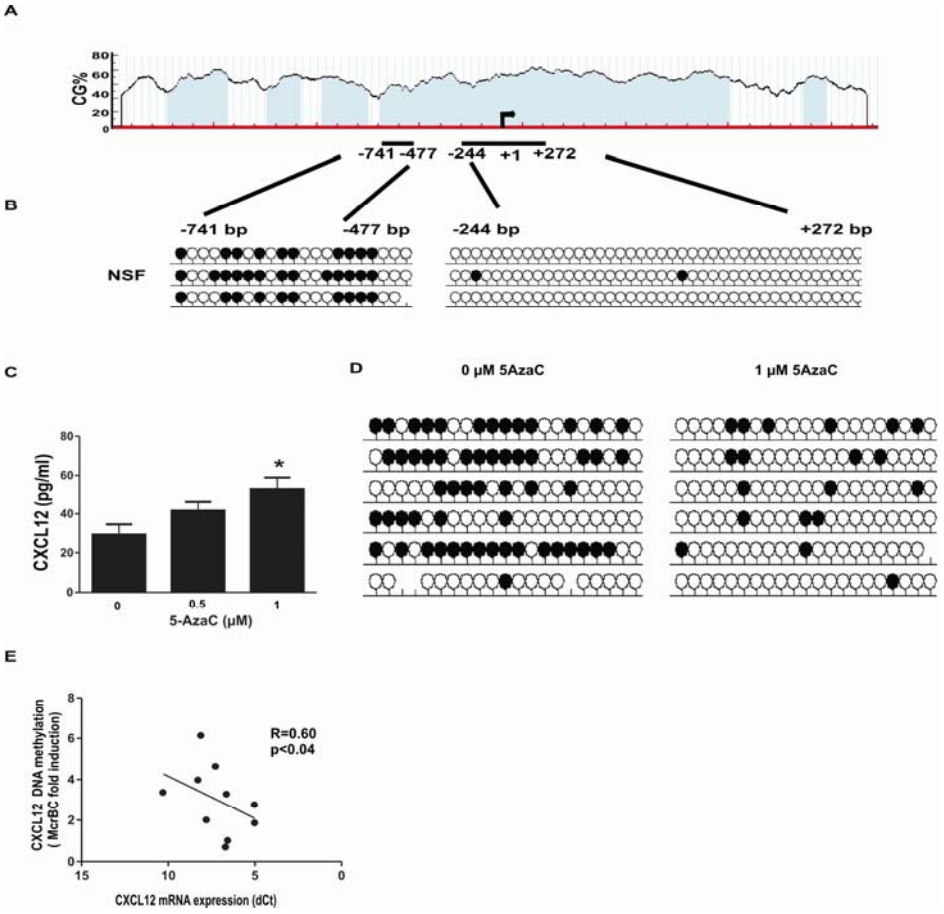

Figure 2. Expression of CXCL12 is regulated by DNA methylation.

A) Computational analysis of the CXCL12 promoter region. (blue coloured regions: CpG islands) The percentage of C and G nucleotides (CG %) are shown 2000 basepairs upstream of the transcription initiation site (arrow) and exon 1. Bisulfite sequencing primers were designed to analyse region -741bp to -477bp and -244bp to +272bp of the CXCL12 promoter. B) Methylated CpG dinucleotides were found in the region -741bp to -477 of NSF (n=1). Each circle represents a methylated (black) or unmethylated (white) CpG dinucleotide. Every row represents a different clone. C) After treatment of OASF with 0 , 0.5 and 1 μM 5-azaC for 6 days, amounts of CXCL12 were determined by ELISA (n=4). 5-azaC treatment induced dose dependent release of CXCL12 (* $p < 0.05$). D) 5-azaC demethylates the analysed promoter region as shown by bisulfite sequencing (n=1 OASF, 6 clones were sequenced). E) McrBC digestion and CXCL12 promoter (-741bp to -477bp) quantitative PCR of RASF (n=11) genomic DNA. Negative correlation between CXCL12 mRNA expression and CXCL12 promoter DNA methylation was shown in RASF.

3.3 Demethylation of CXCL12 promoter upregulates CXCL12 mRNA expression.

To investigate whether the methylation status of the CXCL12 promoter is altered in fibroblasts of RA patients, we compared the percentage of CpG methylation between RASF NSF, OASF and normal lung fibroblasts (Fig. 3A, B). First, a group of 4 pooled RASF patients and a group of 3 pooled OASF patients were compared. The analyzed promoter region of CXCL12 had significantly less CpGs methylated in the RASF group than in the OASF group (RASF 21% ± 7.2 versus OASF 42% ±7.8). We then analysed whether the results from the pooled groups would be reflected in individual patient's analysis. CXCL12 promoter had similar changes as seen in the pooled samples (RASF 1-2 27% ± 4.8 versus OASF 1-2 46% ± 5.6). The CXCL12 promoter in NSF cell cultures was strongly methylated (NSF1 54% and NSF2 60%). As expected, the CXCL12 promoter in lung fibroblasts was similarly strongly methylated as in NSF or OASF. The above results suggest that normally the expression of CXCL12 is repressed by methylation in fibroblasts, but that an epigenetic defect in synovial fibroblasts of RA patients causes an intrinsically increased expression of CXCL12.

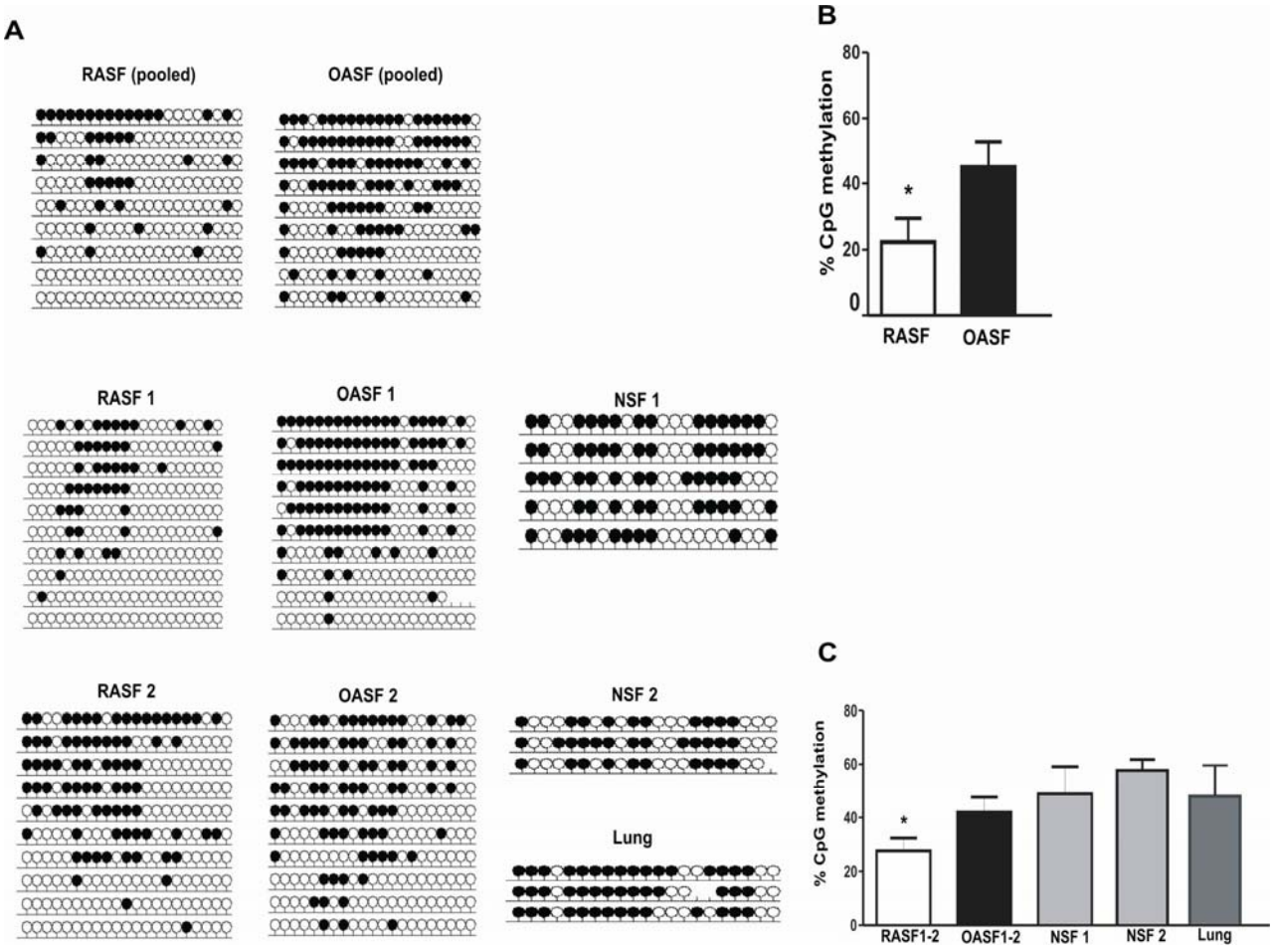

Figure 3. RASF have low levels of CXCL12 promoter DNA methylation.

A) Bisulfite sequencing of CXCL12 promoter region (-741 to -477) in RASF, OASF, NSF and lung fibroblasts. Nine to ten clones were sequenced from two independent RASF and OASF patients or from a pooled group of four RASF and three OASF patients. In addition, three to six clones were sequenced from two NSF and one normal lung fibroblast culture. B) Summary of the data as percentage of CpG methylation between pooled fibroblasts cell types shows that RASF have significantly lower percentage of DNA methylation than OASF. C) Summary of the data as percentage of CpG methylation between individual fibroblasts cell types shows that RASF have significantly lower percentage of DNA methylation than OASF. The methylation percentage was calculated in B and C separately for each clone and the average of total number of clones was plotted in the histogram graph. * $p < 0.005$.

3.4 Overexpression of CXCL12 induces functional changes in RASF

Stimulation of synovial fibroblasts, chondrocytes and osteoclasts with CXCL12 in vitro was previously found to induce the expression of matrix degrading enzymes [16, 27, 28]. Since demethylation induces CXCL12 overexpression, we measured mRNA expression of the collagenases MMP-1 and MMP-13, the gelatinases MMP-2 and MMP-9, stromelysin (MMP-3), and the matrix-bound MMP-14 after stimulation with CXCL12 for 24h in RASF. In addition, we looked at the mRNA expression of the tissue inhibitors of matrix metalloproteinases (TIMPs) -1, -2, and -3. CXCL12 levels in joints of RA patients have been measured to be on average 375 and 750 ng/ml respectively [16, 29]. We stimulated RASFs in vitro with 100 ng/ml. Stimulation with CXCL12 selectively and significantly increased the expression of MMP-1 by 6 ±3 fold, of MMP-3 by 2 ±0.2 fold and of MMP-13 by 4 ±1 fold (Fig.4A). In contrast, CXCL12 neither influenced the expression of MMP-2, MMP-9, or MMP-14, nor did it change the expression levels of TIMPs.

To see whether endogenous production of CXCL12 by RASFs in culture would be enough to stimulate the expression of MMPs, we increased the concentration of CXCL12 in the cell culture supernatants by increasing the ratio between seeded cells and medium and by prolonging incubation times and added an CXCL12 neutralizing antibody or IgG control (Fig. 4B). Expression of MMP-1 was decreased 26% after 24h, and 42% after 96h incubation by CXCL12 blockade compared to IgG control.

We then addressed the question whether CXCL12 signals via CXCR4 or the newly found CXCL12 receptor CXCR7 in synovial fibroblasts. Transcription of the CXCR4 gene produces 4 different mRNAs which putatively encode 4 different isoforms of the protein. We analyzed the expression of these 4 transcripts in PBMCs, synovial fibroblasts, synovial tissues and HeLa cells. None of the tested synovial fibroblasts expressed any transcripts for CXCR4. In PBMCs all four transcripts were amplified, whereas HeLa cells expressed transcript 1, 2 and 4. In RA synovial tissues transcripts 1 and 2 were detectable, probably due to the infiltration of lymphocytes, since in OA synovial tissues none of the transcripts was found (Fig.4C). In contrast to CXCR4, CXCR7 protein was constitutively expressed in synovial fibroblasts (Fig.4D).

We then silenced the expression of CXCR7 with siRNA (Fig.5A) and examined whether this would influence the induction of MMPs in RASF after stimulation with CXCL12. After transfection of siRNA targeting CXCR7, no increase in the mRNA expression of MMP-1, MMP-3 and MMP-13 was seen anymore after stimulation with CXCL12 (Fig.5B). From these

experiments we conclude that CXCR7 is functionally important in synovial fibroblasts and its activation by CXCL12 leads to expression of MMP-1, MMP-3 and MMP-13.

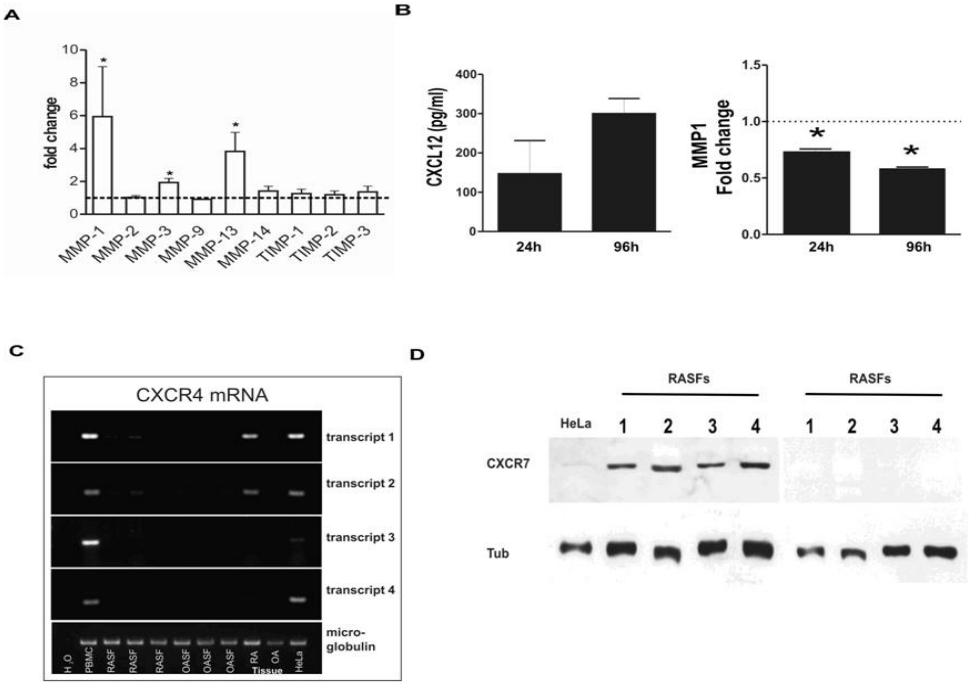

Figure 4. Overexpression of CXCL12 induces MMPs expression. A) Expression of MMP-1, MMP-3 and MMP-13 transcripts are significantly increased after stimulation of RASF with CXCL12 (n = 6). Data are presented as x fold change after stimulation relative to unstimulated cells. B) Time dependent release of CXCL12 protein in cell culture supernatants and inhibition of MMP1 expression using a CXCL12 blocking antibodies (n=3) C) Expression of the four known CXCR4 mRNA transcripts 1-4 by conventional RT-PCR. D) Western blot showing the expression of CXCR7 protein in HeLa cells and RASF obtained from 4 different patients; patient's samples were loaded twice on a 10% gel and the membrane was cut. The left side was incubated with anti-CXCR7 antibodies, the right side with anti-CXCR7 antibodies pre-incubated with CXCR7 synthetic peptide; α-tubulin (tub) served as a loading control.

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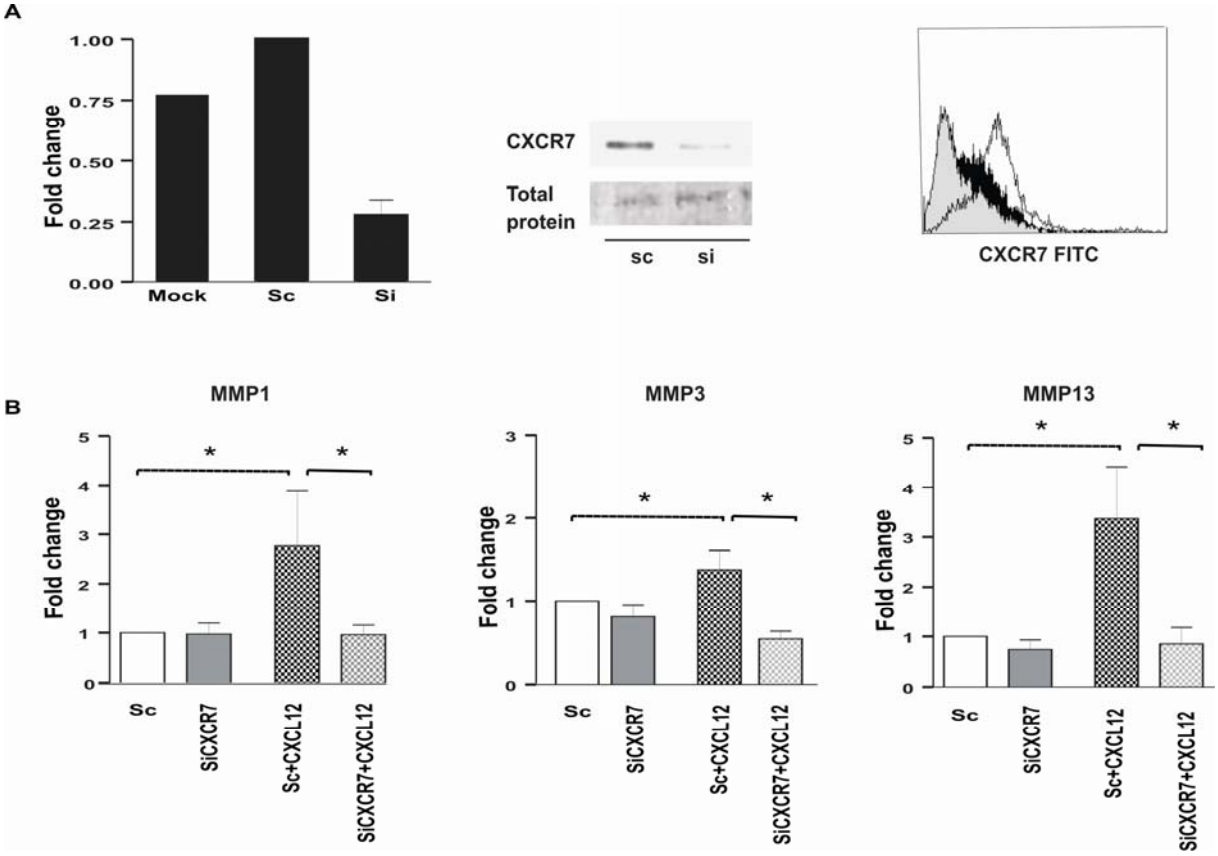

Figure 5. CXCR7 is functional in SF. A) Expression of CXCR7 after silencing was measured on mRNA level (left panel) and on protein level (right panels). Western blot and FACS analysis confirmed silencing of CXCR7 after transfection with siRNA when compared to cells transfected with control siRNA. Grey filled line: rabbit IgG; black filled line: CXCR7 siRNA transfected RASF; black open line: scrambled control siRNA transfected RASF. B) MMP-1, MMP-3 and MMP-13 levels in control transfected RASF (sc) and CXCR7 silenced RASF (siCXCR7) with and without CXCL12 stimulation (n = 7). All values are the mean and SEM.

4. Discussion

In the current paper we show that the high expression of CXCL12 in RASF is due to an epigenetic alteration and that high amount of CXCL12, as found in the joints of RA patients lead to increased expression of MMPs and to joint destruction.

In cancer, both DNA hypomethylation and hypermethylation have been shown to occur [30, 31]. The promoters of p16 or the DNA repair genes MLH1 and BRCA1 for instance have been shown to be silenced by increased DNA methylation in cancer cells [32]. Global hypomethylation was correlated with demethylation of repetitive sequences e.g. Sata, D4Z4, NBL2 and transposable elements e.g. LINE-1 [33, 34]. We and others have reported global hypomethylation of synovial fibroblasts in RA and CD4 T cells in SLE [2, 35]. We also showed that this global hypomethylation affects the promoter of LINE-1 transposable element in RASF and that continuous treatment of NSF with 5-azaC induced changes in gene expression that resemble the activated status of RASF such as increased production of MMP-14, CD29 and cathepsins [2]. Also in SLE, hypomethylation of specific gene promoters have been shown to contribute to the pathogenesis of the disease [35]. Similar to the approach of our current study it was shown that normal T cells treated with 5-azaC upregulated LFA1, CD70 and perforin and that the CpG islands in the promoters of these genes were hypomethylated in T cells of SLE patients [36-38]. DNA methylation of promoter sequences can change the binding of transcription factors. Transcriptional regulation studies of the CXCL12 promoter revealed the presence of E-box consensus sequences which are binding sites for multiple transcription factors such as bHLH, NFAT, SP1 and HIF1α. [39-41]. The low methylation levels observed in the CXCL12 promoter in RASF could change the binding of these transcription factors and thereby increase gene expression. In healthy fibroblasts, the CXCL12 promoter was strongly methylated in our study. In cancers, it has been shown that DNA hypermethylation of the CXCL12 promoter in colon carcinomas, mammary carcinomas and the MCF7 breast cancer cell line inhibits tumor metastasis.

In the current study basal levels of CXCL12 were higher in RASF compared to OASF. Even though these changes were not dramatic, we believe that they are of clinical significance since CXCL12 expression positively correlated with levels of CRP. CXCL12 is expressed at high levels in RA, and it has been implicated in a number of pathogenic events such as recruitment and persistence of inflammatory cells in the synovium, as well as production of cytokines and

matrix degrading enzymes [16, 17, 20, 28, 42]. The role of CXCL12 in cartilage destruction was further supported by in vivo experiments which showed that CXCL12 antagonists inhibit fibroblast-induced cell infiltration in immunodeficient mice [43]. CXCL12 can signal via CXCR4 and CXCR7. Expression of CXCR4 has been detected in synovium of healthy individuals as well as in OA and RA patients [20, 44]. Controversial data is published regarding the expression of CXCR4 in synovial fibroblasts. Whereas Garcia-Vicuna et al detected CXCR4 in synovial fibroblasts, Kanbe et al did not [16, 28]. We also could not detect mRNA for CXCR4 in synovial fibroblasts of RA or OA patients. Since, pancreatic cancer cells do not express CXCR4 due to promoter hypermethylation, CXCR4 silencing could also occur by DNA methylation in synovial fibroblasts. CXCR7, previously named RDC1 was first classified as an orphan receptor since no ligand could be found [45]. Recent publications have then related this receptor to cell recruitment, migration and proliferation after binding of CXCL12 in various cell types [9, 46, 47]. In the present study, we show that functional CXCR7 is also expressed in synovial fibroblasts and that it plays a role in the induction of MMP production via CXCL12. Based on our data we hypothesize that while CXCR4 might be responsible for CXCL12 mediated cell trafficking into RA joints, CXCR7 mediates CXCL12 signaling in resident synovial fibroblasts. The exact mechanism of signaling after binding of CXCL12 to CXCR7 is not clear yet [9, 46]. Recent studies found an association between CXCR7 and the adaptor protein β-arrestin2 [48-50]. Interactions of arrestins with different receptors have been shown to facilitate activation of the MAPK cascade, a signaling pathway which is known to regulate also MMPs [51].

High production of MMPs by RASFs has been identified as major mechanism of joint destruction in RA [52]. In particular, MMP-1 and MMP-13 were reported to play a crucial role in the invasive properties of RASF as shown in the SCID mouse co-implantation model [53, 54]. Expression of MMPs is regulated by multiple pathways. DNA hypomethylation modulates expression of different MMPs in cancer cells. In OA chondrocytes the adipocytokine leptin has not only been found to be upregulated by promoter DNA hypomethylation but also to stimulate expression of MMP-13 [55]. Also, a variety of cytokines and chemokines stimulate the expression of MMPs [56, 57]. The chemokine CXCL12 was reported to induce the expression of MMP-3 in chondrocytes and of MMP-9 in osteoclasts [16, 27]. Furthermore, increased gelatinase and collagenase activity was described after CXCL12 stimulation in synovial fibroblasts [28]. Our present data suggest that the increased collagenase activity after CXCL12 stimulation stems from increased transcription

of MMP-1 and MMP-13. On the other hand, CXCL12 stimulation neither changed the quantity of MMP-2 nor of MMP-9 transcripts, pointing to indirect regulation of gelatinase activity by CXCL12. Since MMP-3 was described to contribute to the activation of pro-MMP-9 and we found that MMP-3 is more abundant in CXCL12 stimulated cells, it is probable that CXCL12 increases gelatinase activity via upregulation of MMP-3 [58, 59]. Based on the differential induction of specific MMPs after stimulation with CXCL12 it can be concluded that CXCL12 does not induce MMPs via the Activator Protein-1 (AP-1), since all of the measured MMPs contain an AP-1 site in their promoter regions. Differences among the MMP promoters have been described and include not only binding sites for different transcription factors but also variability in the number and arrangement of binding sites, which strongly increases the complexity of MMP regulation [60].

In summary, our data show that basal expression of the chemokine CXCL12 is increased in RASF due to a defect in gene regulation by DNA methylation. In RA joints, accumulated CXCL12 produced by RASF might lead to increased expression of MMPs which mediate joint destruction. In addition to the previously reported global hypomethylation, we identified CpG island specific hypomethylation of CXCL12 which is involved in the intrinsic activation of RASF and thereby in the perpetuation of RA.

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Chapter 4: Conclusion

For the first time, the methylation status of cells has been analysed in the synovial tissues of patients with rheumatoid arthritis (RA). RA synovial tissues were found to have hypomethylated nuclei (Chapter 2, Figure 1). Especially synovial fibroblasts had low amounts of 5-methylcytosine. RASF had hypomethylated repetitive sequences, such as the LINE-1 promoter / 5'-UTR. Since no genetic polymorphism can explain the hyperactive phenotype of synovial fibroblasts to date, we hypothesised that the epigenetic modifications, particularly DNA methylation, can cause the activation of synovial fibroblasts and lead to joint destruction. When normal synovial fibroblasts were treated over a long period of time with the DNMT1 inhibitor 5-azacytidine (5-azaC), they overexpressed genes that are associated with joint destruction, such as matrix metalloproteinases, intergrins and others summarized in Chapter 2 (Suppl. Tables 1,2). These cells mimic RASF largely.

DNA methylation has been extensively studied in cancer [1]. However, deregulation of the DNA methylation has also been observed in autoimmune [2], developmental [3] and psychiatric diseases [4]. In SLE, a global hypomethylation occurs in CD4 T cells [5]. Mice treated with 5-azaC developed autoreactive CD4 T cells that resemble lupus [6]. Bisulfite sequencing identified that the gene promoter of the CD11a integrin gene in lupus T cells and CD70 - the B-cells costimulatory molecule - in CD4 T cells from lupus patients were hypomethylated [7, 8]. Furthermore, global hypomethylation was described in SLE monozygotic discordant twins, the ribosomal genes being mostly affected by the hypomethylation of CpG islands [9].

Deregulation of DNMTs has been suggested to cause global hypomethylation in various diseases. For example, T cells from SLE patients and monozygotic twins have reduced expression of DNMT1 [9, 10]. In RASF, we reported that the protein expression ratio of DNMT1 / proliferation nuclear antigen (PCNA) was decreased. In addition, pro-inflammatory cytokines further reduced the ratio of DNMT1/PCNA in stimulated cells. However, decreased DNMT1 may not be the only factor responsible for global DNA hypomethylation. A detailed investigation of the cellular DNA methylation pathway revealed a complex interplay between different enzymes and other proteins, such as methyl-binding proteins [11]. In addition, DNA methylation is linked to other metabolic pathways involving S-adenosylmethionine (SAM), i.e. the cells' methyl donor. For example, two specific enzymes are involved in the formation of methionine, the precursor of SAM, and both can have an important influence on DNA

methylation, namely 1) methionine synthase (MS) that converts homocysteine into methionine, and 2) methylenetetrahydrofolate reductase (MTHFR) that catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-Methylene-THF) to 5-methyltetrahydrofolate (5-Methyl-THF), i.e. the cosubstrate for homocysteine remethylation to methionine (Figure 1).

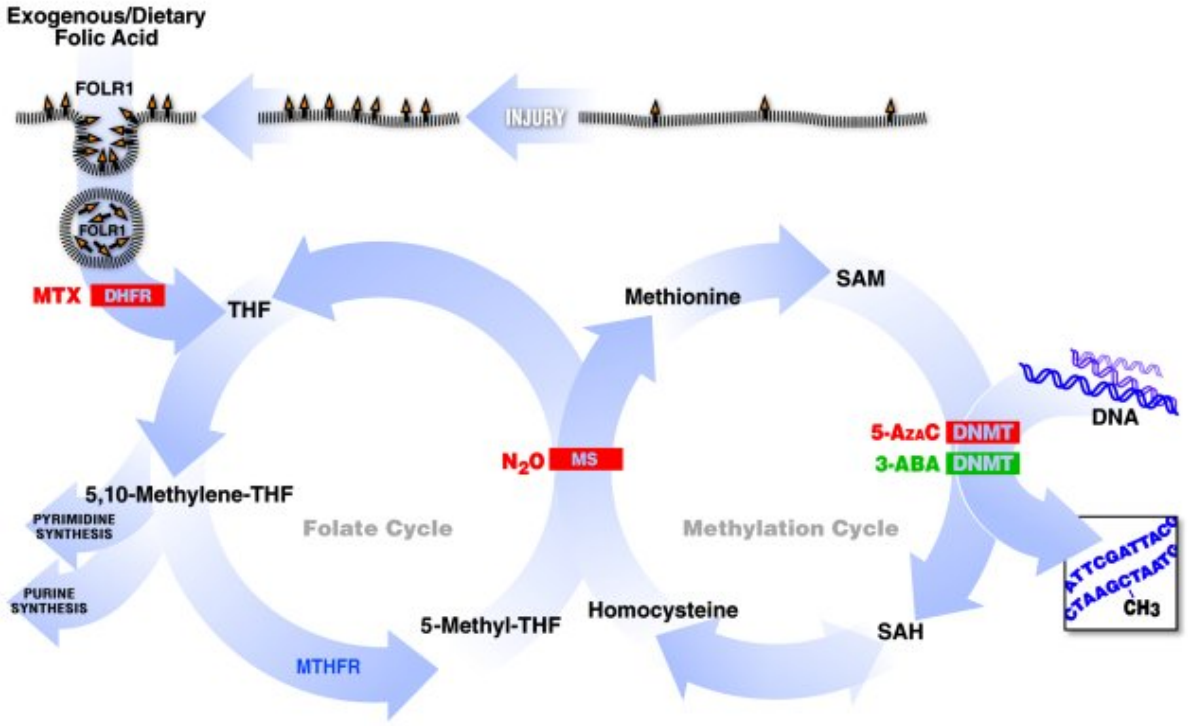

Figure 1: The folate and methylation pathways [12].

The enzyme MTHFR is associated with the genetic mutation C677T in vascular diseases and RA [13, 14]. The mutation causes inhibition of its enzymatic function that alters its enzymatic activity and affects global DNA methylation [15]. Individuals homozygous to the mutation have high levels of homocysteine in their blood and lower amounts of DNA methylation. Mice with the MTHFR mutation developed hyperhomocysteinemia when these animals were fed with low or high methionine diet [16]. Abnormal high homocysteine levels occur in RA patients [17] and this has been associated with cardiovascular diseases [18]; thus, it would be important to investigate whether homocysteine levels are associated to polymorphisms of the MTHFR gene. Genotypic analysis of MTHFR has to be performed in the hypomethylated RASF. Global DNA hypomethylation was strongly correlated with elevated levels of

homocysteine in patients with uraemia [19]. Therefore, the possible association between a defect in the folate / methylation cycles and global DNA hypomethylation in RASF should be explored.

Another hypothesis for the cause of global hypomethylation is a high rate of SAM consumption in RASF. SAM is also consumed by the cells during the synthesis of polyamines (putrescine, spermidine and spermine) [20]. The enzyme spermidine/spermine-N1-acetyltransferase (SSAT1) recycles spermine and spermidine into putrescine [21]. The expression of this enzyme is upregulated with proinflammatroy cytokines such as IL-1β in RASF [22]. Furthermore, levels of putrescine are found elevated in the synovial fluids of RA. The urinary polyamines were significantly elevated in RA patients compared to controls [23]. Therefore, an overexpression of SSAT1 may result in an increased catabolism of polyamines and/or increased recycling of polyamines into putrescine, and in parallel is associated to a decreased cellular level of SAM. This hypothesis has to be proven experimentally.

Reversal of DNA hypomethylation may reduce the activity of RASF and concomitantly of joint destruction. Therefore, a new therapeutic strategy should be developed to reverse DNA hypomethylation. The SCID mouse invasion model with RASF and normal cartilage can be used to investigate whether L-methionine or SAM can reverse RASF-mediated cartilage destruction. Previous research in humans and animals has used dietary supplements of vitamin B12, folate and L-methionine or SAM in the treatment of cancer and neurological disorders [24, 25].

SAM is a natural compound that is not only the methyl donor of the cell, but also has been shown to block the demethylase activity of HEK293 cell and thus promote DNA hypermethylation [26]. Supplementation of breast cancer cells with SAM inhibits growth and metastasis [25]. SAM causes the hypermethylation of genes involved in breast cancer metastasis such as urokinase (uPA), myogenin in muscle cell differentiation and preselin 1 (PSEN1) in amyloidogenesis [24, 25, 27].

Maternal nutrition plays an important role in the regulation of the epigenome as shown in the A^{vy} (yellow agouti) mouse [28]. The agouti gene encodes for a paracrine signalling molecule which causes the yellow colour in the hair follicles of the agouti mouse. The gene expression of the agouti gene is regulated by an IAP transposable element upstream of its transcriptional

start site. IAP elements are usually methylated in the genome. In the agouti mouse, the degree of methylation in IAP mice varies dramatically, causing different coat colours. Supplementation of the female agouti mouse with methyl donating substances 2 weeks before pregnancy and during pregnancy alters the epigenome and colour of their offsprings [29]. Overall, the above observations in different mouse models and in disease supports that dietary supplementation with folic acid, vitamin B12 and L-methionine may reverse the methyl group metabolism and have a beneficial role in the treatment of RA patients. The possible beneficial effect of high methionine supplementation diets should be investigated in various animal models of arthritis.

Finally, the characterisation of a specific gene target that was hypothesized to have altered DNA methylation in RASF was shown in Chapter 3. The activated RASF secrete chemokines that attract a variety of inflammatory cells into the joint. CXCL12 (SDF-1α) is a chemokine overexpressed by RASF. Our study reported that the promoter of CXCL12 is more hypomethylated in RASF than in normal OASF. The upregulation of CXCL12 can stimulate RASF, via the receptor CXCR7, to produce more matrix metalloproteinases (MMPs).

Studies in RASF by other groups have shown that the apoptosis related death receptor 3 (DR3) gene is hypermethylated and might be related with apoptosis resistance [30]. In addition, other investigators studied how interleukin-6 (IL-6) - a pro-inflammatory cytokine - is regulated by promoter DNA methylation in RA [31]. They found a specific CpG site to be hypomethylated in the promoter of the IL-6 gene in RA lymphocytes. Furthermore, the expression of EphrinB1 has been reported to be increased due to promoter hypomethylation in RASF [32]. In the future, a genome wide DNA methylation analysis using methylation immunoprecipitation and CpG island promoter tilling arrays should be performed comparing fibroblasts of different tissues and synovial fibroblasts of various patients [33]. The promoters of these genes that show differential methylation patterns can be then correlated with animal RA models and provide useful data for the pathogenesis of RA.

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Curriculum Vitae

PERSONAL DETAILS

Name: Emmanuel Karouzakis

Address: Altwiesenstrasse 124, 8051 Zürich, Switzerland

Telephone numbers: Mobile:0041 774185321 Work: 0041 44 255 5866,

Email: emmanuel.karouzakis@usz.ch

Nationality: Greek

EDUCATION

UNIVERSITY OF ZURICH **September 2005-Present**

PhD/Doctorate (Framework Program (FP)6 EU-funded integrated research project: Marie Curie Actions, Research Training Network, Title: "Functional Genomic Approaches Targeting Arthritis": Epigenetic Deregulation in Rheumatoid Arthritis Synovial Fibroblasts. Project Supervisors: Prof Steffen Gay / Dr Michel Neidhart.

IMPERIAL COLLEGE LONDON **October 2000-September 2001**

MSc in Molecular Medicine

Master Project Title: Role of Androgen Receptor Cofactors in Prostate Cancer (Lab based: duration 6 months)

UNIVERSITY OF WOLVERHAMPTON **September 1997-June 2000**

BSc (Honours) Biological Sciences (Molecular Biology and Genetics)
Upper Second Class Degree

PROFESSIONAL EXPERIENCE

St. George's Medical School **May 2003-August 2005**
Professor Angus Dagleish / Dr Hardev Pandha
Medical Oncology

Research Assistant
Project: Dendritic Cells Cancer Vaccine Immunotherapy

Imperial College London **October 2001- December 2002**
Professor Christopher Rudd / Dr Helga Schneider
T cell signaling Lab

Research Assistant
Project: Role of ADAP and its binding to VASP in T cell and Antigen Presenting Cell (APC) conjugate formation.

ORIGINAL PEER REVIEWED PUBLICATIONS

1. Karouzakis E., Rengel Y., Jüngel A., Kolling C., Gay R.E. , Michel B.A. , Tak P.P. , Gay S., Neidhart M., Ospelt C. DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. Genes & Immunity (2011) in press.

2. Stanczyk J., Ospelt C., Karouzakis E., Filer A., Raza K., Kolling C., Gay R., Buckley C.D., Tak P.P., Gay S., Kyburz D. Altered expression of miR-203 in rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation. Arthritis & Rheumatism 2010 Oct 27.

3. Neidhart M., Karouzakis E., Schumann G.G., Gay R.E., Gay S. Trex-1 deficiency in rheumatoid arthritis synovial fibroblasts. Arthritis & Rheumatism 2010 Sep; 62(9):2673-9.

4. Maciejewska-Rodrigues H., Karouzakis E. , Striethol S., Hemmatazad H., Neidhart M., Ospelt C., Gay R.E., Michel B.A., Pap T., Gay S., Jüngel A. Epigenetics and rheumatoid arthritis: The role of SENP1 in the regulation of MMP-1 expression. Journal of Autoimmunity 2010 Aug;35(1):15-22.

5. Karouzakis E., Gay R.E., Gay S. Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. Arthritis & Rheumatism 2009; 60(12):3613-22.

REVIEWS

1. Karouzakis E., Gay R.E., Gay S., Neidhart M. Epigenetic control in rheumatoid arthritis synovial fibroblasts. Nature Reviews Rheumatology 2009; 5(5):266-72.

2. Karouzakis E., Jungel A., Gay R.E. , Kolling C. , Gay S. , Neidhart M. Epigenetic Approaches to the Study of the pathogenesis of Rheumatic Disease. European Musculoskeletal Review 3, 41-43 (2008).

3. Karouzakis E., Neidhart M., Gay R.E., Gay S. Molecular and cellular basis of rheumatoid arthritis joint destruction. Immunology Letters 2006 ;106(1):8-13

POSTER PRESENTATIONS

1. 6-11/11/2010 American College of Rheumatology (ACR) San Francisco, USA [abstract #357] Arthritis & Rheumatism 62, S150 (2010).

2. 16-19/9/2010 The European League Against Rheumatism (EULAR), Rome, Italy

3. 24-29/10/2008 American College of Rheumatology (ACR) San Francisco, USA, Arthritis & Rheumatism 58, S514 (2008).

4. 10-15/11/2006 American College of Rheumatology (ACR) Boston, USA , Arthritis & Rheumatism 54, S213, (2006).

5. 22/10/2006 2nd Symposium of the Zurich Center for Integrative Human Physiology; Epigenetic profile of synovial fibroblasts in patients with rheumatoid arthritis.

ORAL PRESENTATIONS

1. 07/2009 The European League Against Rheumatism (EULAR), Copenhagen, Denmark: [abstract #OP-0227] Ann Rheum Dis 68(Suppl III), 68 (2009).

2. Teaching lecture: “Introduction to Epigenetics”, Center of Experimental Rheumatology University Hospital Zurich, Zurich, Switzerland.

3. EURO-RA research grant, Final Review Meeting 10/2008, Stockholm, Sweden.

4.06/2008 The European League Against Rheumatism (EULAR), Paris, France, [abstract #OP-0110]. Ann Rheum Dis 67(Suppl II), 84 (2008).
5. 24-29/10/2008 American College of Rheumatology (ACR) San Francisco, USA ACR/EULAR Academic Exchange program: ACR study group session.
6.11/11/2007 American College of Rheumatology (ACR) Washington, USA[abstract #745] Arthritis & Rheumatism 56, S317 (2007).
7. EURO-RA research grant, Midterm Review Meeting 16/1/2007, London, UK.

COURSES

1.Eukaryotic Gene Expression, July 17-August 7, 2007, Cold Spring Harbor Laboratory, New York, USA (Duration: 3 weeks/Theory/Lab work)
2. 'Introductory Course in Laboratory Animal Science' LTK Module 1E.
3. Postgraduate Courses of the Zurich Center for Integrative Human Physiology (ZIHP); Introduction to Human Physiology: Milieu interieur/Homeostasis, Oxygen and Movement, Scientific Writing (2 days Theory and practical courses)
4. Radioactivity Training
5. Biosafety Training
6. International Summer School on Cancer Immunology and Immunotherapy, Sept. 8-13, 2003, Ionian Village, Greece.

REFERENCES

Prof. Dr. Michel Neidhart
Center of Experimental
Rheumatology
Gloriastr. 25
CH-8091 Zürich
Phone 0041-44-255-5866
Fax 0041-44-255-4170
E-mail michel.neidhart@usz.ch

Prof. Dr. med. Steffen Gay
Director,
Center of Experimental
Rheumatology
Gloriastr. 25
CH-8091 Zürich
Phone 0041-44-255-57-37
Secretary 0041-44-255-29-62
Fax 0041-44-255-41-70
E-mail steffen.gay@usz.ch

Publication List

Karouzakis E., Rengel Y., Jüngel A., Kolling C., Gay R.E. , Michel B.A. , Tak P.P. , Gay S., Neidhart M., Ospelt C. DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. Genes & Immunity (2011) in press. (Impact factor: 4.222)

Stanczyk J., Ospelt C., Karouzakis E., Filer A., Raza K., Kolling C., Gay R.E., Buckley C.D., Tak P.P., Gay S., Kyburz D. Altered expression of microRNA-203 in rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation. Arthritis & Rheumatism 2011 Feb; 63(2):373-81. (Impact factor: 7.332)

Neidhart M., Karouzakis E., Schumann G.G., Gay R.E., Gay S. Trex-1 deficiency in rheumatoid arthritis synovial fibroblasts. Arthritis & Rheumatism 2010 Sep; 62(9): 2673-9. (Impact factor: 7.332)

Maciejewska-Rodrigues H., Karouzakis E., Strietholt S., Hemmatazad H., Neidhart M., Ospelt C., Gay R.E., Michel B.A., Pap T., Gay S., Jüngel A. Epigenetics and rheumatoid arthritis: the role of SENP1 in the regulation of MMP-1 expression. Journal of Autoimmunity 2010 Aug; 35(1):15-22. (Impact factor: 7.231)

Karouzakis E., Gay R.E., Gay S. Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. Arthritis & Rheumatism 2009; 60(12):3613-22. (Impact factor: 7.231)

Karouzakis E., Gay R.E., Gay S., Neidhart M. Epigenetic control in rheumatoid arthritis synovial fibroblasts. Nature Reviews Rheumatology 2009; 5(5):266-72. (Impact factor: 5.533)

Karouzakis E., Jungel A., Gay R.E. , Kolling C. , Gay S. , Neidhart M. Epigenetic Approaches to the Study of the pathogenesis of rheumatic disease. European Musculoskeletal Review 3, 41-43 (2008).

Karouzakis E., Neidhart M., Gay R.E., Gay S. Molecular and cellular basis of rheumatoid arthritis joint destruction. Immunology Letters 2006; 106(1):8-13. (Impact factor: 2.906)

Book Chapter:

Karouzakis E., Gay R.E., Gay S., Neidhart M. Epigenetic deregulation in rheumatoid arthritis Advances in Experimental Medicine and Biology 2011; 711:137-149

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